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(54) Title: PEPTIDE INHIBITORS OF SELECTIN BINDING

(57) Abstract

The present invention provides novel peptides having as their core region portions of the 109-118 amino acid sequence of P-selectin , E-selectin or L-selectin. The invention also provides pharmaceutical compositions comprising the peptides of the invention, and diagnostic and therapeutic methods utilizing the peptides and pharmaceutical compositions of the invention.

- 1 -

PEPTIDE INHIBITORS OF SELECTIN BINDING

Background of the Invention

This invention relates to peptides which inhibit binding of selectins such as P-selectin, E-selectin and L-
5 selectin.

The adherence of platelets and leukocytes to vascular surfaces is a critical component of the inflammatory response and is part of a complex series of reactions involving the simultaneous and interrelated activation of the
10 complement, coagulation, and immune systems.

The complement proteins collectively play a leading role in the immune system, both in the identification and in the removal of foreign substances and immune complexes, as reviewed by Muller-Eberhard, H.J., Ann. Rev. Biochem. 57:
15 321-347 (1988). Central to the complement system are the C3 and C4 proteins, which when activated covalently attach to nearby targets, marking them for clearance. In order to help control this process, a remarkable family of soluble and membrane-bound regulatory proteins has evolved, each of which
20 interacts with activated C3 and/or C4 derivatives. The coagulation and inflammatory pathways are regulated in a coordinate fashion in response to tissue damage. For example, in addition to becoming adhesive for leukocytes, activated endothelial cells express tissue factor on the cell
25 surface and decrease their surface expression of thrombomodulin, leading to a net facilitation of coagulation reactions on the cell surface. In some cases, a single

- 2 -

receptor can be involved in both inflammatory and coagulation processes.

Leukocyte adherence to vascular endothelium is a key initial step in migration of leukocytes to tissues in response to microbial invasion. Although a class of inducible leukocyte receptors, the CD11-CD18 molecules, are thought to have some role in adherence to endothelium, mechanisms of equal or even greater importance for leukocyte adherence appear to be due to inducible changes in the endothelium itself.

Activated platelets have also been shown to interact with both neutrophils and monocytes *in vitro*. The interaction of platelets with monocytes may be mediated in part by the binding of thrombospondin to platelets and monocytes, although other mechanisms have not been excluded. The mechanisms for the binding of neutrophils to activated platelets are not well understood, except that it is known that divalent cations are required. In response to vascular injury, platelets are known to adhere to subendothelial surfaces, become activated, and support coagulation. Platelets and other cells may also play an important role in the recruitment of leukocytes into the wound in order to contain microbial invasion.

Endothelium exposed to "rapid" activators such as thrombin and histamine becomes adhesive for neutrophils within two to ten minutes, while endothelium exposed to cytokines such as tumor necrosis factor and interleukin-1 becomes adhesive after one to six hours. The rapid endothelial-dependent leukocyte adhesion has been associated with expression of the lipid mediator platelet activating factor (PAF) on the cell surface, and presumably, the appearance of other endothelial surface receptors. The slower cytokine-inducible endothelial adhesion for leukocytes is mediated, at least in part, by E-selectin that is synthesized by endothelial cells after exposure to cytokines and then transported to the cell surface, where it binds neutrophils. The isolation, characterization and cloning of

- 3 -

E-selectin or ELAM-1 is reviewed by Bevilacqua, et al., in
Science 243, 1160-1165 (1989). L-selectin, a peripheral
lymph node homing receptor, also called "the murine Mel 14
antigen", "Leu 8", the "Leu 8 antigen" and "LAM-1", is
5   another structure on neutrophils, monocytes, and lymphocytes
that binds lymphocytes to high endothelial venules in
peripheral lymph nodes. The characterization and cloning of
the protein is reviewed by Lasky, et al., _Cell_ 56, 1045-1055
(1989) (mouse) and Tedder, et al., _J. Exp. Med._ 170, 123-133
10 (1989).

P-selectin, also known as GMP-140 (granule membrane
protein 140), or PADGEM, is a cysteine-rich and heavily
glycosylated integral membrane glycoprotein with an apparent
molecular weight of 140,000 as assessed by sodium dodecyl
15 sulfate polyacrylamide gel electrophoresis (SDS-PAGE). P-
selectin was first purified from human platelets by McEver
and Martin, _J. Biol. Chem._ 259: 9799-9804 (1984). The
protein is present in alpha granules of resting platelets but
is rapidly redistributed to the plasma membrane following
20 platelet activation, as reported by Stenberg, et al., (1985).
The presence of P-selectin in endothelial cells and its
biosynthesis by these cells was reported by McEver, et al.,
Blood 70(5) Suppl. 1:355a, Abstract No. 1274 (1987). In
endothelial cells, P-selectin is found in storage granules
25 known as the Weibel-Palade bodies. (McEver, et al. _J. Clin._
Invest. 84: 92-99 (1989) and Hattori, et al., _J. Biol. Chem._
264: 7768-7771 (1989)). P-selectin (called GMP-140 or
PADGEM) has also been reported to mediate the interaction of
activated platelets with neutrophils and monocytes by Larsen,
30 et al., in _Cell_ 59, 305-312 (October 1989) and Hamburger and
McEver, _Blood_ 75: 550-554 (1990).

The cDNA-derived amino acid sequence, reported by
Johnston, et al., in _Cell_ 56, 1033-1044 (March 24 1989), and
in U.S. Serial No. 07/320,408 filed March 8, 1989, indicates
35 that it contains a number of modular domains that are likely
to fold independently. Beginning at the N-terminus, these
include a "lectin" domain, an "EGF" domain, nine tandem

- 4 -

consensus repeats similar to those in complement binding
proteins, a transmembrane domain (except in a soluble form
that appears to result from differential splicing), and a
cytoplasmic tail.

5   When platelets or endothelial cells are activated
by mediators such as thrombin, the membranes of the storage
granules fuse with the plasma membrane, the soluble contents
of the granules are released to the external environment, and
membrane bound P-selectin is presented within seconds on the
10 cell surface. The rapid redistribution of P-selectin to the
surface of platelets and endothelial cells as a result of
activation suggested that this glycoprotein could play an
important role at sites of inflammation or vascular
disruption.

15   This important role has been confirmed by the
observation that P-selectin is a receptor for neutrophils
(Geng et al., Nature 343:757-760 (1990); Hamburger and
McEver, Blood 75:550-554 (1990)), monocytes (Larsen, et al.
Cell 59:305-312 (1989)); Moore, et al., J. Cell Biol.
20 112:491-499 (1991)), and perhaps a subset of lymphocytes
(Moore, et al. J. Cell Biol. 112:491-499 (1991)). Thus, GMP-
140 can serve as a receptor for leukocytes following its
rapid mobilization to the surfaces of platelets and
endothelial cells stimulated with agonists such as thrombin.
25 This role in leukocyte recruitment may be important in
hemostatic and inflammatory processes in both physiologic and
pathologic states.

        Peptides derived from P-selectin are described in
U.S. Serial No. 07/554,199 entitled "Functionally Active
30 Selectin-Derived Peptides" filed July 17, 1990 by Rodger P.
McEver that are useful in diagnostics and in modulating the
hemostatic and inflammatory responses in a patient wherein a
therapeutically effective amount of a peptide capable of
blocking leukocyte recognition of P-selectin is administered
35 to the patient. U.S. Serial No. 07/554,199 filed July 17,
1990, also discloses that peptide sequences within the lectin
domain of P-selectin, having homology with the lectin domains

- 5 -

of other proteins, especially E-selectin and the L-selectin, selectively inhibit neutrophil adhesion to purified P-selectin, and can therefore be used in diagnostic assays of patients and diseases characterized by altered binding by
5   these molecules, in screening assays for compounds altering this binding, and in clinical applications to inhibit or modulate interactions of leukocytes with platelets or endothelial cells involving coagulation and/or inflammatory processes.
10   E-selectin, L-selectin, and P-selectin have been termed "selectins", based on their related structure and function. E-selectin is not present in unstimulated endothelium. However, when endothelium is exposed to cytokines such as tumor necrosis factor of interleukin-1, the
15   gene for E-selectin is transcribed, producing RNA which in turn is translated into protein. The result is that E-selectin is expressed on the surface of endothelial cells one to four hours after exposure to cytokines, as reported by Bevilacqua et al., Proc.Natl.Acad.Sci.USA 84: 9238-9242
20   (1987) (in contrast to P-selectin, which is stored in granules and presented on the cell surface within seconds after activation). E-selectin has been shown to mediate the adherence of neutrophils to cytokine-treated endothelium and thus appears to be important in allowing leukocytes to
25   migrate across cytokine-stimulated endothelium into tissues. The cDNA-derived primary structure of E-selectin indicates that it contains a "lectin" domain, an EGF domain, and six (instead of the nine in P-selectin) repeats similar to those of complement-regulatory proteins, a transmembrane domain,
30   and a short cytoplasmic tail. There is extensive sequence homology between P-selectin and E-selectin throughout both proteins, but the similarity is particularly striking in the lectin and EGF domains.
          Homing receptors are lymphocyte surface structures
35   that allow lymphocytes to bind to specialized endothelial cells in lymphatic tissues, termed high endothelial cells or high endothelial venules (reviewed by Yednock and Rosen,

- 6 -

<u>Advances in Immunology</u>, vol. 44, F.I. Dixon, ed., 313-378
(Academic Press, New York 1989). This binding allows
lymphocytes to migrate across the endothelium into the
lymphatic tissues where they are exposed to processed
5   antigens. The lymphocytes then re-enter the blood through
the lymphatic system. L-selectin, a lymphocyte homing
receptor, contains a lectin domain, an EGF domain, two
complement-binding repeats, a transmembrane domain, and a
short cytoplasmic tail. L-selectin also shares extensive
10 sequence homology with P-selectin, particularly in the lectin
and EGF domains.

        Based on a comparison of the lectin domains between
P-selectin, E-selectin, and L-selectin, it may be possible to
select those peptides inhibiting binding of neutrophils to P-
15 selectin which will inhibit binding of E-selectin, L-
selectin, and other homologous selectins, to components of
the inflammatory process, or, conversely, which will inhibit
only P-selectin binding.

        The *in vivo* significance of platelet-leukocyte
20 interactions has not been studied carefully. However, in
response to vascular injury, platelets are known to adhere to
subendothelial surfaces, become activated, and support
coagulation. Platelets and other cells may also play an
important role in the recruitment of leukocytes into the
25 wound in order to contain microbial invasion. Conversely,
leukocytes may recruit platelets into tissues at sites of
inflammation, as reported by Issekutz, et al., <u>Lab. Invest.</u>
49:716 (1983).

        The coagulation and inflammatory pathways are
30 regulated in a coordinate fashion in response to tissue
damage. For example, in addition to becoming adhesive for
leukocytes, activated endothelial cells express tissue factor
on the cell surface and decrease their surface expression of
thrombomodulin, leading to a net facilitation of coagulation
35 reactions on the cell surface. In some cases, a single
receptor can be involved in both inflammatory and coagulation
processes.

- 7 -

Proteins involved in the hemostatic and inflammatory pathways are of interest for diagnostic purposes and treatment of human disorders. However, there are many problems using proteins therapeutically. Proteins are

5 usually expensive to produce in quantities sufficient for administration to a patient. Moreover, there can be a reaction against the protein after it has been administered more than once to the patient. It is therefore desirable to develop peptides having the same, or better, activity as the

10 protein, which are inexpensive to synthesize, reproducible and relatively innocuous.

It is preferable to develop peptides which can be prepared synthetically, having activity at least equal to, or greater than, the peptides derived from the protein itself.

15 It is therefore an object of the present invention to provide peptides interacting with cells recognized by selectins, including P-selectin, E-selectin, and L-selectin.

It is another object of the present invention to provide methods for using these peptides to inhibit leukocyte

20 adhesion to endothelium or to platelets.

It is a further object of the present invention to provide methods for using these peptides to modulate the immune response and the hemostatic pathway.

It is yet another object of the present invention

25 to provide peptides for use in diagnostic assays relating to P-selectin, E-selectin and L-selectin.

Summary of the Invention

This invention relates to novel peptides having as their core region portions of the 109-118 amino acid sequence

30 of P-selectin, E-selectin or L-selectin. More specifically, this invention relates to novel peptides of Formulas I and II:
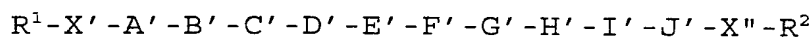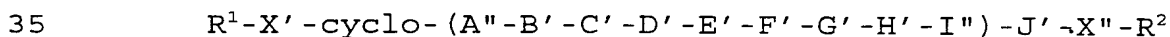
$$R^1-X'-A'-B'-C'-D'-E'-F'-G'-H'-I'-J'-X''-R^2$$

(I)

35 $$R^1-X'-cyclo-(A''-B'-C'-D'-E'-F'-G'-H'-I'')-J'-X''-R^2$$

(II)

or pharmaceutically acceptable salts thereof, wherein:

X' is an N-terminus amino acid linear sequence of from zero to 10 amino acids, and R^1 is a moiety attached to the terminal α amino group of X', or the terminal α-amino
5 group of the adjacent amino acid if X is zero;

X" is a C-terminus amino acid linear sequence of from zero to 10 amino acids, and R^2 is a moiety attached to the carboxyl carbon of X" or the carboxyl carbon of the adjacent amino acid if X" is zero;

10 A' is null (signifying no amino acid) or D- or L-cysteine;

A" is D- or L-cysteine;

B' is D- or L-histidine, D- or L-serine, D- or L-leucine, D- or L-phenylalanine, D- or L-asparagine, D- or L-
15 proline or D- or L-glutamine;

C' is D- or L-lysine, D- or L-histidine, D- or L-arginine, or D- or L-serine;

D' is D- or L-lysine, D- or L-leucine, D- or L-alanine, D- or L-phenylalanine, D- or L-histidine, D- or L-
20 arginine, or D- or L-serine;

E' is D- or L-lysine, D- or L-phenylalanine, D- or L-glutamine, or D- or L-arginine;

F' is D- or L-histidine, D- or L-leucine, D- or L-alanine, D- or L-isoleucine, D- or L-threonine, or D- or L-
25 arginine;

G' is D- or L-alanine, D- or L-phenylalanine, D- or L-histidine, D- or L-isoleucine, or D- or L-glutamine;

H' is D- or L-leucine, D- or L-phenylalanine, D- or L-isoleucine, D- or L-proline, or D- or L-alanine;

30 I' is D- or L-cysteine, D- or L-phenylalanine, D-or L-isoleucine, D- or L-histidine, D- or L-leucine, D- or L-valine, D- or L-threonine, or D- or L-serine;

I" is D- or L-cysteine;

J' is D- or L-tyrosine, D- or L-phenylalanine, D-
35 or L-isoleucine, or D- or L-valine;

- 9 -

R¹ is hydrogen (signifying a free N-terminal group), lower alkyl, aryl, formyl, alkanoyl, aroyl, alkyloxycarbonyl or aryloxycarbonyl;

R² is OH (signifying a free C-terminal carboxylic
5 acid), OR³, signifying ester, where R³ is lower alkyl or aryl or R² is NR⁵R⁶ where R⁵ and R⁶ are each selected independently from hydrogen, lower alkyl, aryl or cyclic alkyl.

The peptides of Formulas I and II have as their core region the 109-118 amino acid sequence of the selectins.
10 Residue 1 is defined as the N-terminus of the mature protein after the cleavage of the signal peptide.

The peptides of Formulas I and II should inhibit the binding of neutrophils to P-selectin in concentrations of peptide ranging from about 10 to about 1500 μM. Tests also
15 indicate that alterations within the core sequence, as well as N-terminal and C-terminal flanking regions, do not result in loss of biological activity.

This invention relates not only to the novel peptides of Formulas I and II, but also to pharmaceutical
20 compositions comprising them, to diagnostic and therapeutic methods utilizing them, and to methods of preparing them.

Detailed Description of the Invention

Preferred peptides of this invention are those of Formula I and II wherein, together or independently: R¹ is
25 hydrogen or acetyl (Ac) and R² is OH or NH₂. More preferred are those peptides wherein R² is NH₂.

One group of preferred peptides includes those of Formula I where, independently, A' is null, B' is Phe, His, Leu, Asn or Ser; C' is Lys or Arg; D' is Lys, Phe, Leu, Ala;
30 E' is Lys or Arg; F' is Leu or Arg; G' is Ala; H' is Leu; I' is Cys, Ile or Phe, and J' is Tyr.

Test results have indicated that peptides in which E' is Arg have superior activity. Accordingly, a more preferred group of peptides are those in which E' is Arg.
35 Specifically preferred peptides include the following:

- 10 -

(SEQ ID NO:1)   Cys-Leu-Lys-Lys-Lys-His-Ala-Leu-Cys-Tyr-
                     NH₂;

(SEQ ID NO:2)   Cys-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-
                     NH₂;

5   (SEQ ID NO:3)   Cys-His-Lys-Leu-Lys-Ala-Ala-Leu-Cys-Tyr-
                     NH₂;

(SEQ ID NO:4)   cyclo-(Cys-Leu-Lys-Lys-Lys-His-Ala-Leu-
                     Cys)-Tyr-NH₂;

(SEQ ID NO:5)   cyclo-(Cys-Ser-Lys-Lys-Lys-Leu-Ala-Leu-
10   Cys)-Tyr-NH₂;

(SEQ ID NO:6)   cyclo-(Cys-His-Lys-Leu-Lys-Ala-Ala-Leu-
                     Cys)-Tyr-NH₂;

(SEQ ID NO:7)   Ac-Phe-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-
                     NH₂;

15 (SEQ ID NO:8)   Phe-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-NH₂;

(SEQ ID NO:9)   Ac-His-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-
                     NH₂;

(SEQ ID NO:10)   His-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-NH₂;

(SEQ ID NO:11)   Leu-Lys-Lys-Lys-His-Ala-Leu-Cys-Tyr-NH₂;

20 (SEQ ID NO:12)   Ac-Leu-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-
                     NH₂;

(SEQ ID NO:13)   Leu-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:14)   Ac-Asn-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-
                     NH₂;

25 (SEQ ID NO:15)   Asn-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:16)   Pro-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:17)   Gln-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:18)   Ser-His-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:19)   Ac-Ser-Lys-Ala-Lys-Leu-Ala-Leu-Cys-Tyr-
30   NH₂;

(SEQ ID NO:20)   Ser-Lys-Phe-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:21)   Ser-Lys-His-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:22)   Ser-Lys-Lys-Phe-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:23)   Ac-Ser-Lys-Lys-Lys-Ala-Ala-Leu-Cys-Tyr-
35   NH₂;

- 11 -

(SEQ ID NO:24)   Ser-Lys-Lys-Lys-Ala-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:25)   Ser-Lys-Lys-Lys-His-ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:26)   Ser-Lys-Lys-Lys-Ile-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:27)   Ser-Lys-Lys-Lys-Leu-Ala-Phe-Cys-Tyr-NH$_2$;

5 (SEQ ID NO:28)   Ser-Lys-Lys-Lys-Leu-Ala-Ile-Cys-Tyr-NH$_2$;

(SEQ ID NO:29)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Phe-NH$_2$;

(SEQ ID NO:30)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Ile-NH$_2$;

(SEQ ID NO:31)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:32)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Phe-Tyr-NH$_2$;

10 (SEQ ID NO:33)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Ile-Val-NH$_2$;

(SEQ ID NO:34)   Ac-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Ile-Tyr-NH$_2$;

(SEQ ID NO:35)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Ile-Tyr-NH$_2$;

(SEQ ID NO:36)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-His-Tyr-NH$_2$;

15 (SEQ ID NO:37)   Ac-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Leu-Tyr-NH$_2$;

(SEQ ID NO:38)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Leu-Tyr-NH$_2$;

(SEQ ID NO:39)   Ac-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Val-Tyr-NH$_2$;

20 (SEQ ID NO:40)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Val-Tyr-NH$_2$;

(SEQ ID NO:41)   Ser-Lys-Lys-Lys-Leu-Ala-Ley-Thr-Tyr-NH$_2$;

(SEQ ID NO:42)   Ser-Lys-Lys-Lys-Leu-Ala-Pro-Cys-Tyr-NH$_2$;

(SEQ ID NO:43)   Ser-Lys-Lys-Lys-Leu-Phe-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:44)   Ser-Lys-Lys-Lys-Leu-His-Leu-Cys-Tyr-NH$_2$;

25 (SEQ ID NO:45)   Ser-Lys-Lys-Lys-Leu-Ile-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:46)   Ser-Lys-Lys-Lys-Leu-Gln-Ala-Cys-Tyr-NH$_2$;

(SEQ ID NO:47)   Ac-Ser-Lys-Lys-Lys-thr-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:48)   Ser-Lys-Lys-Gln-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

30 (SEQ ID NO:49)   Ac-Ser-Lys-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

- 12 -

(SEQ ID NO:50)   Ser-Lys-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:51)   Ac-Ser-Lys-Leu-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:52)   Ser-Lys-Leu-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

5   (SEQ ID NO:53)   Ac-Ser-Lys-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:54)   Ser-Lys-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:55)   Ac-Ser-Lys-Arg-Lys-Arg-Ala-Leu-Cys-Tyr-NH₂;

10   (SEQ ID NO:56)   Ser-Lys-Arg-Lys-Arg-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:57)   Ac-Ser-Lys-Arg-Arg-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:58)   Ser-Lys-Arg-Arg-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:59)   Ser-Lys-Arg-Arg-Leu-Ala-Leu-Ser-Tyr-NH₂;

15   (SEQ ID NO:60)   Ser-Lys-Ser-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:61)   Ac-Ser-Arg-Ala-Arg-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:62)   Ser-Arg-Ala-Arg-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:63)   Ac-Ser-Arg-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;
20

(SEQ ID NO:64)   Ser-Arg-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:65)   Ac-Ser-Arg-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:66)   Ser-Arg-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-NH₂;

25   (SEQ ID NO:67)   Ser-Arg-Lys-Arg-Leu-Ala-Leu-Ser-Tyr-NH₂;

(SEQ ID NO:68)   Ac-Ser-Arg-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:69)   Ser-Arg-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

(SEQ ID NO:70)   Ser-Ser-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH₂;

30   More preferred peptides are those with Sequence ID
Nos. 2, 6, 8, 9, 12, 15, 20, 31, 33, 35, 49, 50, 51, 52, 53,
54, 55, 56, 57, 58, 61, 62, 63, 64, 65, 66, 68, and 69.

As used herein, the term "alkyl" includes branched, straight-chain, and cyclic saturated hydrocarbons. The term "lower alkyl" means an alkyl having from one to six carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl,
5 isobutyl, t-butyl, pentyl, isopentyl, neopentyl, cyclopentylmethyl and hexyl. The term "alkanoyl" means

$$\overset{\displaystyle O}{\underset{\displaystyle R^7-C-}{\|}}$$

10 wherein R^7 is a alkyl group.
The term "aroyl" means

$$\overset{\displaystyle O}{\underset{\displaystyle R^8-C-}{\|}}$$

15   wherein R^8 is an aryl group. The term "aryl" means an aromatic or heteroaromatic structure having between one and three rings, which may or may not be ring fused structures, and are optionally substituted with halogens, carbons, or other heteroatoms such as nitrogen (N), sulfur
20 (S), phosphorus (P), and boron (B).
The term alkoxycarbonyl means

$$\overset{\displaystyle O}{\underset{\displaystyle R^9-O-C-O-}{\|}}$$
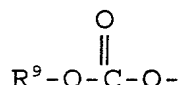
25   wherein R^9 is a lower alkyl group.
The term aryloxycarbonyl means

$$\overset{\displaystyle O}{\underset{\displaystyle R^{10}-O-C-O-}{\|}}$$
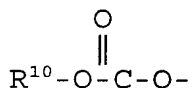
30   wherein R^{10} is an aryl and arylmethyl group.
Halogen refers to fluorine, chlorine, bromine or iodine.
The term "terminal α-amino group of X" refers to the α-amino group of the N-terminal amino acid of X.
35   The peptides of Formulas I and II can be used in the form of the free peptide or a pharmaceutically acceptable salt. Amine salts can be prepared by treating the peptide with an acid according to known methods. Suitable acids include inorganic acids such as hydrochloric acid,
40 hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids

- 14 -

such as formic acid, acetic acid, propionic acid, glycolic
acid, lactic acid, pyruvic acid, oxalic acid, malonic acid,
succinic acid, maleic acid, fumaric acid, anthranilic acid,
cinnamic acid, naphthalenesulfonic acid, and sulfanilic acid.

5   Carboxylic acid groups in the peptide can be
converted to a salt by treating the peptide with a base
according to known methods. Suitable bases include inorganic
bases such as sodium hydroxide, ammonium hydroxide, and
potassium hydroxide, and organic bases such as mono-, di-,

10 and tri-alkyl and aryl amines (e.g., triethylamine,
diisopropylamine, methylamine, and dimethylamine and
optionally substituted mono-, di, and tri-ethanolamines.

        As referred to herein, the amino acid components of
the peptides and certain materials used in their preparation

15 are identified by abbreviations for convenience. These
abbreviations are as follows:

Amino Acid	Abbreviations	
L-alanine	Ala	A
D-alanine	D-Ala	a
L-arginine	Arg	R
5 D-arginine	D-Arg	r
D-asparagine	D-Asn	n
L-asparagine	Asn	N
L-aspartic acid	Asp	D
D-aspartic acid	D-Asp	d
10 L-cysteine	Cys	C
D-cysteine	D-Cys	c
L-glutamic acid	Glu	E
D-glutamic acid	D-Glu	e
L-glutamine	Gln	Q
15 D-glutamine	D-Gln	q
glycine	Gly	G
L-histidine	His	H
D-histidine	D-His	h
L-isolelucine	Ile	I
20 D-isolelucine	D-Ile	i
L-leucine	Leu	L
D-leucine	D-Leu	l
L-lysine	Lys	K
D-lysine	D-Lys	k
25 L-phenylalanine	Phe	F
D-phenylalanine	D-Phe	f
L-proline	Pro	P
D-proline	D-Pro	p
L-serine	Ser	S
30 D-serine	D-Ser	s
L-threonine	Thr	T
D-threonine	D-Thr	t
L-tyrosine	Tyr	Y
D-tyrosine	D-Tyr	y
35 L-tryptophan	Trp	W
D-tryptophan	D-Trp	w
L-valine	Val	V
D-valine	D-Val	v
L-methionine	Met	M
40 D-methionine	D-Met	m

Reagents	Abbreviations
Trifluoroacetic acid	TFA
Methylene chloride	CH_2Cl_2
N,N-Diisopropylethylamine	DIEA
45 N-Methylpyrrolidone	NMP
1-Hydroxybenzotriazole	HOBT
Dimethylsulfoxide	DMSO
Acetic anhydride	Ac_2O
Diisopropylcarbodiimide	DIC
50 Acetic acid	HOAc

- 16 -

Amino acids preceded by L- or D- refer, respectively, to the L- or D- enantiomer of the amino acid, whereas amino acids not preceded by L- or D- refer to the L-enantiomer.

Methods of Preparation of Peptides

5   The peptides can generally be prepared following known techniques, as described, for example, in the cited publications, the teachings of which are specifically incorporated herein. In a preferred method, the peptides are prepared following the solid-phase synthetic technique

10   initially described by Merrifield in J.Amer.Chem.Soc., 85, 2149-2154 (1963). Other techniques may be found, for example, in M. Bodanszky, et al, Peptide Synthesis, second edition, (John Wiley & Sons, 1976), as well as in other reference works known to those skilled in the art.

15   Appropriate protective groups usable in such syntheses and their abbreviations will be found in the above text, as well as in J.F.W. McOmie, Protective Groups in Organic Chemistry, (Plenum Press, New York, 1973). The common protective groups used herein are t-butyloxycarbonyl (Boc),

20   fluorenylmethoxycarboyl (FMOC), benzyl (Bzl), tosyl (Tos), o-bromo-phenylmethoxycarbonyl (BrCBZ), phenylmethoxycarbonyl (CBZ), 2-chloro-phenylmethoxycarbonyl (2-Cl-CBZ), 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr), trityl (Trt), formyl (CHO), and tertiary butyl (t-Bu).

25   General synthetic procedures for the synthesis of peptides of Formula I and II the invention by solid phase methodology are as follows:

- 17 -

A.   **General Synthetic Procedures For Solid Phase Peptide Synthesis Using N^{α}-Boc Protection**

			REPETITIONS	TIME
1.	25% TFA in CH_2Cl_2		1	3 min.
2.	50% TFA in CH_2Cl_2		1	16 min.
3.	CH_2Cl_2		5	3 min.
4.	5% DIEA in NMP		2	4 min.
5.	NMP		6	5 min.
6.	Coupling step		1	57 min.
	a.	Preformed BOC-Amino Acid-HOBT active ester in NMP		37 min.
	b.	DMSO		16 min.
	c.	DIEA		5 min.
7.	10% Ac_2O, 5% DIEA in NMP		1	9 min.
8.	CH_2Cl_2		5	3 min.

B.   **General Synthetic Procedure For Solid Phase Peptide Synthesis Using N^{α}-FMOC Protection**

		REPETITIONS	TIME
1.	50% piperidine in DMF	1	1 min.
2.	50% piperidine in NMP	1	12 min.
3.	NMP	7	1 min.
4.	Coupling	1	71 min.

Amino acid and HOBT in NMP added to the resin followed by the addition of DIC in NMP.

HOBT active ester in NMP or

5.	NMP	1	1 min.
6.	Repeat steps 4-5	1	
7.	NMP	2	1 min.

N-terminal acetylation on the deprotected N^{α}-amino group of peptides synthesized using either Boc or FMOC strategies can be accomplished with 10% Ac_2O and 5% DIEA in NMP, followed by washing of the peptide resin with NMP and/or CH_2Cl_2.

The peptides can also be prepared using standard genetic engineering techniques known to those skilled in the art. For example, the peptide can be produced enzymatically

- 18 -

by inserting nucleic acid encoding the peptide into an
expression vector, expressing the DNA, and translating the
DNA into the peptide in the presence of the required amino
acids.   The peptide is then purified using chromatographic or
5   electrophoretic techniques, or by means of a carrier protein
which can be fused to, and subsequently cleaved from, the
peptide by inserting into the expression vector in phase with
the peptide encoding sequence a nucleic acid sequence
encoding the carrier protein.   The fusion protein-peptide may
10   be isolated using chromatographic, electrophoretic or
immunological techniques (such as binding to a resin via an
antibody to the carrier protein).   The peptide can be cleaved
using chemical methodology or enzymatically, as by, for
example, hydrolases.
15   Peptides of the invention can also be prepared using
solution methods, by either stepwise or fragment
condensations.   An appropriately alpha amino-protected amino
acid is coupled to an appropriately alpha carboxyl protected
amino acid (such protection may not be required depending on
20   the coupling method chosen) using diimides, symmetrical or
unsymmetrical anhydrides, BOP, or other coupling reagents or
techniques known to those skilled in the art.   These
techniques may be either or enzymatic.   The alpha amino
and/or alpha carboxyl protecting groups are removed and the
25   next suitably protected amino acid or block of amino acids
are coupled to extend the growing peptide.   Various
combinations of protecting groups and of chemical and/or
enzymatic techniques and assembly strategies can be used in
each synthesis.
30   The peptides of Formula II are cyclic by virtue of
the formation of a disulfide bond between cysteine residues.
The general techniques for the formation of this bond are
described by G. Barany and R. B. Merrifield in <u>The Peptides
Analysis, Synthesis, Biology</u>, (Academic Press, Inc., 1979),
35   as well as in other reference works known to those skilled in
the art.

Methods of Preparation of Pharmaceutical Compositions

Pharmaceutical compositions of this invention comprise a pharmaceutically acceptable carrier or diluent and an effective quantity of one or more of the peptides of Formula

5 I or II or an acid or base salt thereof. The carrier or diluent may take a wide variety of forms depending on the form of preparation desired for administration, e.g., sublingual, rectal, nasal, oral, or parenteral.

In preparing the compositions in oral dosage form, any

10 of the usual pharmaceutical media may be employed, for example, waters, oils, alcohols, flavoring agents, preservatives, and coloring agents, to make an oral liquid preparation (e.g., suspension, elixir, or solution) or with carriers such as starches, sugars, diluents, granulating

15 agents, lubricants, binders, and disintegrating agents, to make an oral solid preparation (e.g., powder, capsule, or tablet).

Controlled release forms or enhancers to increase bioavailability may also be used. Because of their ease in

20 administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are employed. If desired, tablets may be sugar coated or enteric coated by standard techniques.

For parenteral products, the carrier will usually be

25 sterile water, although other ingredients to aid solubility or as preservatives may be included. Injectable suspensions may also be prepared, in which case appropriate liquid carriers and suspending agents can be employed.

The peptides can also be administered locally at a

30 wound or inflammatory site by topical application of a solution or cream.

Alternatively, the peptide may be administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a

35 patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describes methods for encapsulating biological materials in liposomes. Essentially, the material is

- 20 -

dissolved in an aqueous solution, the appropriate
phospholipids and lipids added, along with surfactants if
required, and the material dialyzed or sonicated, as
necessary. A review of known methods is by G. Gregoriadis,

5   Chapter 14, "Liposomes", Drug Carriers in Biology and
Medicine, pp. 287-341 (Academic Press, 1979). Microspheres
formed of polymers or proteins are well known to those
skilled in the art, and can be tailored for passage through
the gastrointestinal tract directly into the bloodstream.

10 Alternatively, the peptide can be incorporated and the
microspheres, or composite of microspheres, implanted for
slow release over a period of time, ranging from days to
months. See, for example, U.S. Patents Nos. 4,906,474,
4,925,673 and 3,625,214.

15   The peptides are generally active when administered
parenterally in amounts of at least about 1 μg/kg body
weight. Effective doses by other routes of administration
are generally those which result in similar blood level to
i.v. doses of at least about 1 μg/Kg. For treatment to

20 prevent organ injury in cases involving reperfusion, the
peptides may be administered parenterally in amounts from
about 0.01 to about 10 mg/kg body weight. Generally, the
same range of dosage amounts may be used in treatment of
other diseases or of conditions where inflammation is to be

25 reduced. This dosage will be dependent, in part, on whether
one or more peptides are administered. A synergistic effect
may be seen with combinations of peptides from different, or
overlapping, regions of the lectin domain, or in combination
with peptides derived from the EGF domain of P-, E- or L-

30 selectin. For treatment to prevent organ injury in cases
involving reperfusion, the peptides may be administered
parenterally in amounts from about 0.01 to about 10 mg/kg
body weight. Generally, the same range of dosage amounts may
be used in treatment of other diseases or of conditions where

35 inflammation is to be reduced. This dosage will be
dependent, in part, on whether one or more peptides are
administered. A synergistic effect may be seen with

- 21 -

combinations of peptides from different, or overlapping, regions of the lectin domain, or in combination with peptides derived form the EGF domain of P-selectin.

Methods for Demonstrating Binding

5   Peptides that are biologically active are those which inhibit binding of neutrophils, monocytes, subsets of lymphocytes or other cells to P-selectin, or which inhibit leukocyte adhesion to endothelium that is mediated by ELAM-1 and/or the homing receptor.

10   Peptides can be screened for their ability to inhibit adhesion to cells, for example, neutrophil adhesion to purified P-selectin immobilized on plastic wells, using the assay described by Geng, et al., <u>Nature</u> 343, 757-760 (1990).

          Human neutrophils are isolated from heparinized whole
15   blood by density gradient centrifugation on Mono-Poly resolving media, Flow Laboratories. Neutrophil suspensions are greater than 98% pure and greater than 95% viable by trypan blue exclusion. For adhesion assays, neutrophils are suspended at a concentration of 2 x 10^6 cells/mL in Hanks'
20   balanced salt solution containing 1.26 mM Ca^{2+} and 0.81 mM Mg^{2+} (HBSS, Gibco) with g mg/mL human serum albumin (HBSS/HSA). Adhesion assays are conducted in triplicate in 96-well microtiter plates, Corning, incubated at 4°C overnight with 50 microliters of various protein solutions.

25   P-selectin is isolated from human platelet lysates by immunoaffinity chromatography on antibody S12-Sepharose™ and ion-exchange chromatography on a Mono-Q™ column (FLPC, Pharmacia Fine Chemicals), as follows.

          Outdated human platelet packs (100 units) obtained
30   from a blood bank and stored at 4°C are pooled, adjusted to 5mM EDTA at pH 7.5, centrifuged at 4,000 rpm for 30 minutes in 1 liter bottles, then washed three times with 1 liter of 0.1 M NaCl, 20 mM Tris pH 7.5 (TBS), 5 mM EDTA, 5 mM benzamidine.

35   The pellets are then resuspended in a minimum amount of wash buffer and made 1mM in DIFP, then frozen in 50 mL

screwtop tubes at -80°C. The frozen platelets are thawed and resuspended in 50 mL TBS, 5 mM benzamidine, 5 mM EDTA pH 7.5, 100 M leupeptin. The suspension is frozen and thawed two times in a dry ice-acetone bath using a 600 mL lyophilizing
5 flask, then homogenized in a glass/teflon mortar and pestle and made 1 mM in DIFP. The NaCl concentration is adjusted to 0.5 M with a stock solution of 4 M NaCl. After stirring the suspension at 4°C, it is centrifuged in polycarbonate tubes at 33,000 rpm for 60 minutes at 4°C. The supernatant (0.5 M
10 NaCl wash) is removed and saved; this supernatant contains the soluble form of P-selectin. Care is taken not to remove the top part of the pellet with the supernatant. The pellets are then homogenized in extraction buffer (TBS, 5 mM benzamidine, 5 mM EDTA, pH 7.5, 100 μM leupeptin, 2% Triton
15 X-100). After centrifugation at 19,500 rpm for 25 minutes at 4°C, the supernatant is removed. The extraction procedure is repeated with the pellet and the supernatant is combined with the first supernatant. The combined extracts, which contain the membrane form of P-selectin, are adjusted to 0.5 M NaCl.
20   The soluble fraction (0.5 M NaCl wash) and the membrane extract (also adjusted to 0.5 M NaCl) are absorbed with separate pools of the monoclonal antibody S12 (directed to P-selectin) previously coupled to Affigel (Biorad) at 5 mg/mL for 2 hours at 4°C. After letting the resins settle,
25 the supernatants are removed. The S12 Affigel containing bound GMP-140 is then loaded into a column and washed overnight at 4°C with 400 mL of 0.5 M NaCl, 20 mM Tris pH 7.5, 0.01% Lubrol PX.
       Bound P-selectin is eluted from the S12 Affigel with
30 100 mL of 80% ethylene glycol, 1 mM MES pH 6.0, 0.01% Lubrol PX. Peak fractions with absorbance at 280 nm are pooled. Eluates are dialyzed against TBS with 0.05% Lubrol, then applied to a Mono Q column (FPLC from Pharmacia). The concentrated protein is step eluted with 2 M NaCl, 20 mM Tris
35 pH 7.5 (plus 0.05% Lubrol PX for the membrane fraction). Peak fractions are dialyzed into TBS pH 7.5 (plus 0.05% Lubrol PX for the membrane fraction).

- 23 -

P-selectin is plated at 5 micrograms/mL and the
control proteins: human serum albumin (Alb), platelet
glycoprotein IIb/IIIa (IIb), von Willebrand factor (vWF),
fibrinogen (FIB), thrombomodulin (TM), gelatin (GEL) or human
5   serum (HS), are added at 50 micrograms/mL. All wells are
blocked for 2 hours at 22°C with 300 microliters HBSS
containing 10 mg/mL HSA, then washed three times with HBSS
containing 0.1% Tween-20 and once with HBSS. Cells (2 x 10^5
per well) are added to the wells and incubated at 22°C for 20
10   minutes. The wells are then filled with HBSS/HSA, sealed
with acetate tape (Dynatech), and centrifuged inverted at 150
g for 5 minutes. After discarding nonadherent cells and
supernates, the contents of each well are solubilized with
200 microliters 0.5% hexadecyltrimethylammonium bromide,
15   Sigma, in 50 mM potassium phosphate, pH. 6.0, and assayed for
myeloperoxidase activity, Ley, et al., <u>Blood</u> 73, 1324-1330
(1989). The number of cells bound is derived from a standard
curve of myeloperoxidase activity versus numbers of cells.
Under all assay conditions, the cells release less than 5% of
20   total myeloperoxidase and lactate dehydrogenase. Inhibition
is read as a lower percent adhesion, so that a value of 5%
means that 95% of the specific adhesion was inhibited.

Peptides are tested at concentrations between 1.0 mM
to 0.001 mM and a percent inhibition calculated for each
25   concentration. A least squares fit is done on a plot of
peptide concentration versus percent inhibition and an IC_{50}
value calculated. The IC_{50} is defined as the concentration
of peptide that will inhibit 50% of the neutrophil binding to
the P-selectin lawn.

30   Activity data are presented either as an IC_{50} for each
peptide or the percent inhibition at a defined concentration.

Table I gives the IC_{50} values in mM for peptides of the
invention in inhibiting the binding of human neutrophils to
P-selectin.

- 24 -

TABLE I

INHIBITION OF BINDING OF HUMAN NEUTROPHILS TO P-SELECTIN

	Structure		IC_{50} (mM)
5	CLKKKHALCY-NH$_2$	SEQ ID NO:1	0.269
	CSKKKLALCY-NH$_2$	SEQ ID NO:2	0.048
	CHKLKAALCY-NH$_2$	SEQ ID NO:3	0.282
	Cyclo-(CLKKKHALC)-Y-NH$_2$	SEQ ID NO:4	0.626
	Cyclo-(CSKKKLALC)-Y-NH$_2$	SEQ ID NO:5	0.078
10	Cyclo-(CHKLKAALC)-Y-NH$_2$	SEQ ID NO:6	0.002
	Ac-FKKKLALCY-NH$_2$	SEQ ID NO:7	0.074
	FKKKLALCY-NH$_2$	SEQ ID NO:8	0.020
	Ac-HKKKLALCY-NH$_2$	SEQ ID NO:9	0.011
	HKKKLALCY-NH$_2$	SEQ ID NO:10	0.055
15	LKKKHALCY-NH$_2$	SEQ ID NO:11	0.178
	Ac-LKKKLALCY-NH$_2$	SEQ ID NO:12	0.026
	LKKKLALCY-NH$_2$	SEQ ID NO:13	0.065
	Ac-NKKKLALCY-NH$_2$	SEQ ID NO:14	0.053
	NKKKLALCY-NH$_2$	SEQ ID NO:15	0.019
20	PKKKLALCY-NH$_2$	SEQ ID NO:16	0.103
	QKKKLALCY-NH$_2$	SEQ ID NO:17	1.013
	SHKKLALCY-NH$_2$	SEQ ID NO:18	0.362
	Ac-SKAKLALCY-NH$_2$	SEQ ID NO:19	0.060
	SKFKLALCY-NH$_2$	SEQ ID NO:20	0.037
25	SKHKLALCY-NH$_2$	SEQ ID NO:21	0.888
	SKKFLALCY-NH$_2$	SEQ ID NO:22	0.554
	Ac-SKKKAALCY-NH$_2$	SEQ ID NO:23	0.192
	SKKKAALCY-NH$_2$	SEQ ID NO:24	0.232
	SKKKHALCY-NH$_2$	SEQ ID NO:25	0.656
30	SKKKIALCY-NH$_2$	SEQ ID NO:26	0.062
	SKKKLAFCY-NH$_2$	SEQ ID NO:27	0.086
	SKKKLAICY-NH$_2$	SEQ ID NO:28	0.107
	SKKKLALCF-NH$_2$	SEQ ID NO:29	0.065
	SKKKLALCI-NH$_2$	SEQ ID NO:30	0.867
35	SKKKLALCY-NH$_2$	SEQ ID NO:31	0.019

SKKKLALFY-NH₂	SEQ ID NO:32	0.015
SKKKLALIV-NH₂	SEQ ID NO:33	0.390
Ac-SKKKLALIY-NH₂	SEQ ID NO:34	0.128
SKKKLALIY-NH₂	SEQ ID NO:35	0.043
SKKKLALHY-NH₂	SEQ ID NO:36	0.177
Ac-SKKKLALLY-NH₂	SEQ ID NO:37	0.088
SKKKLALLY-NH₂	SEQ ID NO:38	0.131
Ac-SKKKLALVY-NH₂	SEQ ID NO:39	0.164
SKKKLALVY-NH₂	SEQ ID NO:40	0.233
SKKKLALTY-NH₂	SEQ ID NO:41	0.423
SKKKLAPCY-NH₂	SEQ ID NO:42	0.897
SKKKLFLCY-NH₂	SEQ ID NO:43	0.095
SKKKLHLCY-NH₂	SEQ ID NO:44	0.207
SKKKLILCY-NH₂	SEQ ID NO:45	0.095
SKKKLQACY-NH₂	SEQ ID NO:46	0.329
Ac-SKKKTALCY-NH₂	SEQ ID NO:47	0.082
SKKQLALCY-NH₂	SEQ ID NO:48	0.195
Ac-SKKRLALCY-NH₂	SEQ ID NO:49	0.009
SKKRLALCY-NH₂	SEQ ID NO:50	0.004
Ac-SKLKLALCY-NH₂	SEQ ID NO:51	0.012
SKLKLALCY-NH₂	SEQ ID NO:52	0.009
Ac-SKRKLALCY-NH₂	SEQ ID NO:53	0.012
SKRKLALCY-NH₂	SEQ ID NO:54	0.004
Ac-SKRKRALCY-NH₂	SEQ ID NO:55	0.026
SKRKRALCY-NH₂	SEQ ID NO:56	0.024
Ac-SKRRLALCY-NH₂	SEQ ID NO:57	0.008
SKRRLALCY-NH₂	SEQ ID NO:58	0.008
SKRRLALSY-NH₂	SEQ ID NO:59	0.632
SKSKLALCY-NH₂	SEQ ID NO:60	0.858
Ac-SRARLALCY-NH₂	SEQ ID NO:61	0.047
SRARLALCY-NH₂	SEQ ID NO:62	0.034
Ac-SRKKLALCY-NH₂	SEQ ID NO:63	0.017
SRKKLALCY-NH₂	SEQ ID NO:64	0.016
Ac-SRKRLALCY-NH₂	SEQ ID NO:65	0.018
SRKRLALCY-NH₂	SEQ ID NO:66	0.003
SRKRLALSY-NH₂	SEQ ID NO:67	0.586
Ac-SRRKLALCY-NH₂	SEQ ID NO:68	0.027

- 26 -

SRRKLALCY-NH$_2$ SEQ ID NO:69   0.014

SSKKLALCY-NH$_2$ SEQ ID NO:70   0.282

Clinical Applications

Since the selectins have several functions related to
5   leukocyte adherence, inflammation, and coagulation, compounds
which interfere with binding of P-selectin, E-selectin or L-
selectin can be used to modulate these responses.

For example, the peptides can be used to competitively
inhibit leukocyte adherence by competitively binding to P-
10 selectin receptors on the surface of leukocytes. This kind
of therapy would be particularly useful in acute situations
where effective, but transient, inhibition of leukocyte-
mediated inflammation is desirable. Chronic therapy by
infusion of the peptides may also be feasible in some
15 circumstances.

An inflammatory response may cause damage to the host
if unchecked, because leukocytes release many toxic molecules
that can damage normal tissues. These molecules include
proteolytic enzymes and free radicals. Examples of
20 pathological situations in which leukocytes can cause tissue
damage include injury from ischemia and reperfusion,
bacterial sepsis and disseminated intravascular coagulation,
adult respiratory distress syndrome, tumor metastasis,
rheumatoid arthritis and atherosclerosis.
25   Reperfusion injury is a major problem in clinical
cardiology. Therapeutic agents that reduce leukocyte
adherence in ischemic myocardium can significantly enhance
the therapeutic efficacy of thrombolytic agents.
Thrombolytic therapy with agents such as tissue plasminogen
30 activator or streptokinase can relieve coronary artery .
obstruction in many patients with severe myocardial ischemia
prior to irreversible myocardial cell death. However, many
such patients still suffer myocardial neurosis despite
restoration of blood flow. This "reperfusion injury" is
35 known to be associated with adherence of leukocytes to
vascular endothelium in the ischemic zone, presumably in part

because of activation of platelets and endothelium by
thrombin and cytokines that makes them adhesive for
leukocytes (Romson et al., Circulation 67: 1016-1023 (1983)).
These adherent leukocytes can migrate through the endothelium
5   and destroy ischemic myocardium just as it is being rescued
by restoration of blood flow.

        There are a number of other common clinical disorders
in which ischemia and reperfusion results in organ injury
mediated by adherence of leukocytes to vascular surfaces,
10 including strokes; mesenteric and peripheral vascular
disease; organ transplantation; and circulatory shock (in
this case many organs might be damaged following restoration
of blood flow).

        Bacterial sepsis and disseminated intravascular
15 coagulation often exist concurrently in critically ill
patients. They are associated with generation of thrombin,
cytokines, and other inflammatory mediators, activation of
platelets and endothelium, and adherence of leukocytes and
aggregation of platelets throughout the vascular system.
20 Leukocyte-dependent organ damage is an important feature of
these conditions.

        Adult respiratory distress syndrome is a devastating
pulmonary disorder occurring in patients with sepsis or
following trauma, which is associated with widespread
25 adherence and aggregation of leukocytes in the pulmonary
circulation. This leads to extravasation of large amounts of
plasma into the lungs and destruction of lung tissue, both
mediated in large part by leukocyte products.

        Two related pulmonary disorders that are often fatal
30 are in immunosuppressed patients undergoing allogeneic bone
marrow transplantation and in cancer patients suffering from
complications that arise from generalized vascular leakage
resulting from treatment with interleukin-2 treated LAK cells
(lymphokine-activated lymphocytes). LAK cells are known to
35 adhere to vascular walls and release products that are
presumably toxic to endothelium. Although the mechanism by
which LAK cells adhere to endothelium is now known, such

- 28 -

cells could potentially release molecules that activate
endothelium and then bind to endothelium by mechanisms
similar to those operative in neutrophils.

Tumor cells from many malignancies (including
5   carcinomas, lymphomas, and sarcomas) can metastasize to
distant sites through the vasculature. The mechanisms for
adhesion of tumor cells to endothelium and their subsequent
migration are not well understood, but may be similar to
those of leukocytes in at least some cases. The association
10   of platelets with metastasizing tumor cells has been well
described, suggesting a role for platelets in the spread of
some cancers. Recently, it was reported that P-selectin
binds to tumor cells in a variety of human carcinoma tissue
sections (colon, lung, and breast), and that P-selectin binds
15   to the cell surface of a number of cell lines derived from
various carcinomas, but not from melanomas. Aruffo, A., et
al., Proc. Natl. Acad. Sci. USA, 89, 2292-2296 (1992).
Aruggo et al. also reference earlier work suggesting that E-
selectin might be involved in tumor metastasis by mediating
20   the adhesion of a colon carcinoma cell line (HT-20) to
activated endothelial cells in vitro. Platelet-leukocyte
interactions are believed to be important in atherosclerosis.
Platelets might have a role in recruitment of monocytes into
atherosclerotic plaques; the accumulation of monocytes is
25   known to be one of the earliest detectable events during
atherogenesis. Rupture of a fully developed plaque may not
only lead to platelet deposition and activation and the
promotion of thrombus formation, but also the early
recruitment of neutrophils to an area of ischemia.
30   Another area of potential application is in the
treatment of rheumatoid arthritis.

The criteria for assessing response to therapeutic
modalities employing these peptides, and, hence, effective
dosages of the peptides of this invention for treatment, are
35   dictated by the specific condition and will generally follow
standard medical practices. For example, the criteria for
the effective dosage to prevent extension of myocardial

infarction would be determined by one skilled in the art by
looking at marker enzymes of myocardial necrosis in the
plasma, by monitoring the electrocardiogram, vital signs, and
clinical response. For treatment of acute respiratory
5   distress syndrome, one would examine improvements in arterial
oxygen, resolution of pulmonary infiltrates, and clinical
improvement as measured by lessened dyspnea and tachypnea.
For treatment of patients in shock (low blood pressure), the
effective dosage would be based on the clinical response and
10 specific measurements of function of vital organs such as the
liver and kidney following restoration of blood pressure.
Neurologic function would be monitored in patients with
stroke. Specific tests are used to monitor the functioning
of transplanted organs; for example, serum creatinine, urine
15 flow, and serum electrolytes in patients undergoing kidney
transplantation.

Diagnostic Reagents

The peptides can also be used for the detection of
human disorders in which the ligands for the selectins might
20 be defective. Such disorders would most likely be seen in
patients with increased susceptibility to infections in which
leukocytes might not be able to bind to activated platelets
or endothelium. Cells to be tested, usually leukocytes, are
collected by standard medically approved techniques and
25 screened. Detection systems include ELISA procedures,
binding of radiolabeled antibody to immobilized activated
cells, flow cytometry, or other methods known to those
skilled in the art. Inhibition of binding in the presence
and absence of the lectin domain peptides can be used to
30 detect defects or alterations in selectin binding. For
selectins, such disorders would most likely be seen in
patients with increased susceptibility to infections in which
leukocytes would have defective binding to platelets and
endothelium because of deficient leukocyte ligands for P-
35 selectin.

The peptide is labeled radioactively, with a
fluorescent tag, enzymatically, or with electron dense
material such as gold for electron microscopy. The cells to
be examined, usually leukocytes, are incubated with the
5 labeled peptides and binding assessed by methods described
above with antibodies to P-selectin, or by other methods
known to those skilled in the art. If ligands for P-, E- or
L-selectin are also found in the plasma, they can also be
measured with standard ELISA or radioimmunoassay procedures,
10 using labeled P-, E- or L-selectin-derived peptide instead of
antibody as the detecting reagent.

The peptides can also be useful in *in vivo* imaging of
concentrations of cells bearing selectin ligands. Cells
expressing selectin ligands whose abnormally high local
15 concentrations or presence within the body such as cancer
cells, is indicative of a disorder can be imaged using
labeled peptides. These labels may be either intrinsic or
extrinsic to the structure of the specific selectin peptide
and may include, but not be limited to high energy emitters
20 such as ^{111}In or non-radioactive dense atoms to enhance x-ray
contrast.

The following examples are presented to illustrate,
not limit, the invention. In the examples and throughout the
specification, parts are by weight unless otherwise
25 indicated.

**EXAMPLE I: Cyclo-(cystinyl-leucyl-lysyl-lysyl-lysyl-
histidyl-alanyl-leucyl-cystinyl)-tyrosine-amide**

The peptide was prepared on an ABI Model 431A Peptide
Synthesizer using Version 1.12 of the standard BOC software.
30 4-methyl benzhydrylamine resin (0.625 g, 0.5 mmol) was used
in the synthesis. The final weight of the resin was 1.84 g.

The peptide was cleaved from the resin (1.8 g) using
18 mL of HF and 1.8 mL of anisole for 60 min at 0°C. The
resin was washed with ether and the peptide extracted with
35 TFA/DCM (1:1, v/v) (3 x 15 mL) to give 720 mg of crude
peptide. The crude linear peptide (500 mg) was dissolved in
80 mL of 50% HOAc and then added dropwise to the mixture of

water (1200 mL), NH$_4$OH (to keep pH approx. 7.5) and 0.01 M
K$_3$Fe(CN)$_6$ (approx. 3 mL). After each addition of the linear
precursor to the reaction mixture (approx. 1.5 mL) the pH was
adjusted to 7.5 by addition of NH$_4$OH followed by addition of
5   more K$_3$Fe(CN)$_6$ solution. The total volume of the 0.01 M
K$_3$Fe(CN)$_6$ solution used for oxidation was 40 mL. An
additional 2 mL of K$_3$Fe(CN)$_6$ solution was added extra and the
mixture was stirred over 20 min (pH = 7.5), then the pH was
adjusted to 4-5 by addition of HOAc followed by stirring with
10 5 g of anion exchange AG 3-X4, 200-400 mesh, free base form
(Bio-Rad) over 30 min. The resin was filtered off, washed
with 5% acetic acid (3 x 100 mL) and combined fractions
(approx. 1700 mL) were loaded onto a Vydac C-18 column (15μ,
10 X 30 cm) eluting with 0-15% over 5 min and a 15-55%
15 gradient of 80% ethanol in 0.1% TFA over 55 min at a flow
rate of 120 mL per min. Fractions were collected, analyzed
by HPLC and pure fractions pooled, evaporated to approx. 100
mL and lyophilized to give 146 mg of white solids. Amino
acid analysis: Ala 1.01 (1), Cys 1.60 (2), His 1.03 (1), Leu
20 2.00 (2), Lys 2.96 (3), Tyr 0.72 (1).

EXAMPLE II: **Cyclo-(cystinyl-histidyl-lysyl-leucyl-
                     lysyl-alanyl-alanyl-leucyl-cystinyl)-
                     tyrosine-amide**

The peptide was prepared on an ABI Model 431A Peptide
25 Synthesizer using Version 1.12 of the standard BOC software.
4-methyl benzhydrylamine resin (0.625 g, 0.5 mmol) was used
in the synthesis. The final weight of the resin was 1.66 g.
The peptide was cleaved from the resin (1.6 g) using
16 mL of HF and 1.6 mL of anisole for 60 min at 0°C. The
30 resin was washed with ether and the peptide extracted with
TFA/CH$_2$Cl$_2$ (1:1, v/v) (3 x 15 mL) to give 748 mg of crude
peptide. The crude linear peptide (500 mg) was dissolved in
65 mL of 70% HOAc and then added dropwise to the mixture of
water (1200 mL), NH$_4$OH (to keep pH approx. 7.5) and 0.01 M
35 K$_3$Fe(CN)$_6$ (approx. 3 mL). After each addition of the linear
precursor to the reaction mixture (approx. 1.5 mL) the pH was
adjusted to 7.5 by addition of NH$_4$OH followed by addition of

- 32 -

more $K_3Fe(CN)_6$ solution. The total volume of the 0.01 M
$K_3Fe(CN)_6$ solution used for oxidation was 30 mL. An
additional 2 mL of $K_3Fe(CN)_6$ solution was added extra and the
mixture was stirred over 20 min (pH = 7.5), then the pH was
5 adjusted to 4-5 by addition of HOAc followed by stirring with
5 g of anion exchange AG 3-X4, 200-400 mesh, free base form
(Bio-Rad) over 30 min. The resin was filtered off, washed
with 5% acetic acid (3 x 100 mL) and combined fractions
(approx. 1700 mL) were loaded onto a Vydac C-18 column (15 μ,
10 10 x 30 cm) eluting with a 0-15% over 5 min and 15-55%
gradient of 80% ethanol in 0.1% TFA over 55 min at a flow
rate of 120 mL per min. Fractions were collected, analyzed
by HPLC and pure fractions pooled and lyophilized to give 86
mg of white solid. Amino acid analysis: Ala 2.00 (2), Cys
15 1.55 (2), His 1.00 (1), Leu 2.01 (2), Lys 1.98 (2), Tyr 0.70
(1).
Ellman's test for quantitative determination of SH was
negative.

EXAMPLE III: Cystinyl-leucyl-lysyl-lysyl-lysyl-histidyl-
20 **alanyl-leucyl-cystinyl-tyrosine-amide**
The peptide was prepared on an ABI Model 431A Peptide
Synthesizer using Version 1.12 of the standard BOC software.
4-methyl benzhydrylamine resin (0.625 g, 0.5 mmol) was used
in the synthesis. The final weight of the resin was 1.84 g.
25 The peptide was cleaved from the resin (1.8 g) using
18 mL of HF and 1.8 mL of anisole for 60 min at 0°C. The
resin was washed with ether and the peptide extracted with
TFA/CH_2Cl_2 (1:1, v/v) (3 x 15 mL) to give 720 mg of crude
peptide.
30 The crude peptide (220 mg) was purified on a Vydac C-
18 column (15μ, 5 x 25 cm) eluting with a 15-45% gradient of
80% acetonitrile in 0.1% TFA over 120 min at a flow rate of
15 mL per min. Fractions were collected, analyzed by HPLC
and pure fractions pooled and lyophilized to give 32 mg of
35 white solid. Amino acid analysis: Ala 1.02 (1), Cys 0.88
(2), His 1.06 (1), Leu 1.98 (2), Lys 2.94 (3), Tyr 0.87 (1).

- 33 -

EXAMPLE IV: **Cystinyl-histidyl-lysyl-leucyl-lysyl-alanyl-alanyl-leucyl-cystinyl-tyrosine-amide**

The peptide was prepared on an ABI Model 431A Peptide
5 Synthesizer using Version 1.12 of the standard BOC software.
4-methyl benzhydrylamine resin (0.625 g, 0.5 mmol) was used
in the synthesis. The final weight of the resin was 1.66 g.
The peptide was cleaved from the resin (1.6 g) using
16 mL of HF and 1.6 mL of anisole for 60 min at 0°C. The
10 resin was washed with ether and the peptide extracted with
TFA/CH_2Cl_2 (1:1 v/v) (3 x 15 mL) to give 748 mg of crude
peptide.

The crude peptide (249 mg) was purified in two runs on
a Vydac C-18 column (15μ, 5 x 25cm) eluting with a 5-45%
15 gradient of 80% acetonitrile in 0.1% TFA over 120 min at a
flow rate of 15 mL per min. Fractions were collected,
analyzed by HPLC and pure fractions pooled and lyophilized to
give 53 mg of white solid. Amino acid analysis: Ala 1.97
(2), Cys 0.91, (2), His 1.10 (1), Leu 1.98 (2), Lys 1.95 (2),
20 Tyr 0.74 (1).

EXAMPLE V: **Cyclo-(cystinyl-serinyl-lysyl-lysyl-lysyl-leucyl-alanyl-leucyl-cystinyl-tyrosine-amide**

The peptide was prepared on an ABI Model 431A Peptide
Synthesizer using Version 1.12 of the standard BOC software.
25 4-methyl benzhydrylamine resin (0.625 g, 0.5 mmol) was used
in the synthesis. The final weight of the resin was 1.61 g.
The peptide was cleaved from the resin (1.6 g) using
16 mL of HF and 1.6 mL of anisole for 60 min at 0°C. The
resin was washed with ether and the peptide extracted with
30 TFA/CH_2Cl_2 (1:1, v/v) (3 x 15 mL) to give 680 mg of crude
peptide.

The crude linear peptide (460 mg) was dissolved in 60
mL of 50% HOAc and then added dropwise to the mixture of
water (1200 mL), NH_4OH (to keep pH approximately 7.5) and
35 0.01 M $K_3Fe(CN)_6$ (approximately 3 mL). After each addition
of the linear precursor to the reaction mixture   .
(approximately 1.5 mL) its pH was adjusted to 7.5 by addition

of NH$_4$OH followed by addition of K$_3$Fe(CN)$_6$ solution. The
total volume of the 0.01 M K$_3$Fe(CN)$_6$ solution used for
oxidation was 37 mL. The additional 2 mL of K$_3$Fe(CN)$_6$
solution was added extra and the mixture was stirred over 20
5   min (pH = 7.5), then the pH was adjusted to 4-5 by addition
of HOAc followed by stirring with 5 g of anion exchange AG 3-
X4, 200-400 mesh, free base form (Bio-Rad) over 30 min. The
resin was filtered off, washed with 5% acetic acid (3 x 100
mL) and combined fractions (approximately 1650 mL) were
10 loaded onto a Vydac C-18 column (15μ, 10 x 30 cm) eluting
with a 0-25% over 5 min and 25-55% gradient of 80% ethanol in
0.1% TFA over 55 min at a flow rate of 120 mL per min.
Fractions were collected, analyzed by HPLC and pure fractions
pooled, evaporated (approximately 100 mL) and lyophilized to
15 give 158 mg of white solid. Amino acid analysis: Ala 1.02
(1), Cys 1.67 (2), Leu 1.99 (2), Lys 2.92 (3), Ser 0.77 (1),
Tyr 0.78 (1).

EXAMPLE VI: **Cystinyl-serinyl-lysyl-lysyl-lysyl-leucyl-
                         alanyl-leucyl-cystinyl-tyrosine-amide**
20

     The peptide was prepared on an ABI Model 431A Peptide
Synthesizer using Version 1.12 of the standard BOC software.
4-methyl benzhydrylamine resin (0.625 g, 0.5 mmol) was used
in the synthesis. The final weight of the resin was 1.61 g.
25   The peptide was cleaved from the resin (1.6 g) using
16 mL of HF and 1.6 mL of anisole for 60 min at 0°C. The
resin was washed with ether and the peptide extracted with
TFA/CH$_2$Cl$_2$ (1:1, v/v) (3 x 15 mL) to give 680 mg of crude
peptide.
30   The crude peptide (220 mg) was purified on a Vydac C-
18 column (15μ, 10 x 30 cm) eluting with a 0-30% over 5 min
and 30-60% gradient of 80% ethanol in 0.1% TFA over 50 min at
a flow rate of 120 mL per min. Fractions were collected,
analyzed by HPLC and semi-pure fractions pooled, evaporated
35 (approximately 100 mL) and lyophilized to give 66 mg of semi-
pure product.

- 35 -

The semi-pure peptide (66 mg) was repurified on a
Vydac C-18 column (15μ, 5 x 25 cm) eluting with a 20-50%
gradient of 80% acetonitrile in 0.1% TFA over 120 min at a
flow rate of 15 mL/min. Fractions were collected, analyzed
5 by HPLC and pure fractions pooled and lyophilized to give 25
mg of white solid.

Amino acid analysis: Ala 1.00 (1), Cys 1.71 (2), Leu
2.00 (2), Lys 3.00 (3), Ser 0.72 (1), Tyr 0.75 (1).

- 36 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Heavner, George A.
                 Kruszynski, Marian

    (ii) TITLE OF INVENTION: PEPTIDE INHIBITORS OF SELECTIN
           BINDING

  (iii) NUMBER OF SEQUENCES: 6

   (iv) CORRESPONDENCE ADDRESS:
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                   and Norris
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       (D) STATE: PA
       (E) COUNTRY: USA
       (F) ZIP: 19103

    (v) COMPUTER READABLE FORM:
       (A) MEDIUM TYPE: Floppy disk
       (B) COMPUTER: IBM PC compatible
       (C) OPERATING SYSTEM: PC-DOS/MS-DOS
       (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
       (A) APPLICATION NUMBER:
       (B) FILING DATE:
       (C) CLASSIFICATION:

 (viii) ATTORNEY/AGENT INFORMATION:
       (A) NAME: Elderkin, Dianne B.
       (B) REGISTRATION NUMBER: 28,598
       (C) REFERENCE/DOCKET NUMBER: CCOR-0030

   (ix) TELECOMMUNICATION INFORMATION:
       (A) TELEPHONE: 215-568-3100
       (B) TELEFAX: 215-568-3439

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 10 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:

                    (A) NAME/KEY: Peptide
                    (B) LOCATION: 10
                    (D) OTHER INFORMATION: /label= AMINO MODIFIED
                              /note= "NH2 ATTACHED TO TERMINAL CARBOXYL
                              CARBON"

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

          Cys Leu Lys Lys Lys His Ala Leu Cys Tyr
          1   5   10

     (2) INFORMATION FOR SEQ ID NO:2:

          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 10 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: peptide

          (ix) FEATURE:
                    (A) NAME/KEY: Peptide
                    (B) LOCATION: 10
                    (D) OTHER INFORMATION: /label= AMINO MODIFIED
                              /note= "NH2 ATTACHED TO TERMINAL CARBOXYL
                              CARBON"

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

          Cys Leu Lys Lys Lys His Ala Leu Cys Tyr
          1   5   10

     (2) INFORMATION FOR SEQ ID NO:3:

          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 10 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: peptide

          (ix) FEATURE:
                    (A) NAME/KEY: Peptide
                    (B) LOCATION: 10
                    (D) OTHER INFORMATION: /label= AMINO MODIFIED
   /note= "NH2 ATTACHED TO TERMINAL CARBOXYL
                              CARBON"

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Cys Ser Lys Lys Lys Leu Ala Leu Cys Tyr
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Peptide
        (B) LOCATION: 10
        (D) OTHER INFORMATION: /label= AMINO MODIFIED
                /note= "NH2 ATTACHED TO TERMINAL CARBOXYL
                    CARBON"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Cys His Lys Leu Lys Ala Ala Leu Cys Tyr
1               5                   10
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(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Peptide
        (B) LOCATION: 10
        (D) OTHER INFORMATION: /label= AMINO MODIFIED
                /note= "NH2 ATTACHED TO TERMINAL CARBOXYL
                    CARBON"

    (ix) FEATURE:
        (A) NAME/KEY: Peptide
        (B) LOCATION: 1..9
        (D) OTHER INFORMATION: /label= CYCLO-PEPTIDE
                /note= "RESIDUES 1 THROUGH 9 FORM RING"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Cys Leu Lys Lys Lys His Ala Leu Cys Tyr
1               5                   10
```

  (2) INFORMATION FOR SEQ ID NO:6:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 10 amino acids
          (B) TYPE: amino acid
5   (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (ix) FEATURE:
          (A) NAME/KEY: Peptide
10   (B) LOCATION: 10
          (D) OTHER INFORMATION: /label= AMINO MODIFIED
                  /note= "NH2 ATTACHED TO TERMINAL CARBOXYL
                      CARBON"

     (ix) FEATURE:
15   (A) NAME/KEY: Peptide
          (B) LOCATION: 1..9
          (D) OTHER INFORMATION: /label= CYCLO-PEPTIDE
                  /note= "RESIDUES 1 THROUGH 9 FORM RING"

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

20   Cys Ser Lys Lys Lys Leu Ala Leu Cys Tyr
      1   5   10

WHAT IS CLAIMED IS:

    1.   A peptide of Formula I or II:
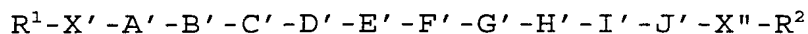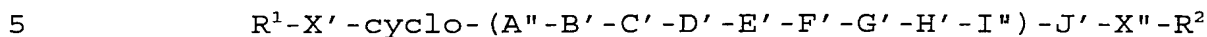
$$R^1-X'-A'-B'-C'-D'-E'-F'-G'-H'-I'-J'-X''-R^2$$

(I)

$$R^1-X'-cyclo-(A''-B'-C'-D'-E'-F'-G'-H'-I'')-J'-X''-R^2$$

(II)

or pharmaceutically acceptable salts thereof, wherein:

    X' is an N-terminus amino acid linear sequence of from zero to 10 amino acids, and R^1 is a moiety attached to the terminal α amino group of X', or the terminal α-amino group of the adjacent amino acid if X is zero;

    X" is a C-terminus amino acid linear sequence of from zero to 10 amino acids, and R^2 is a moiety attached to the carboxyl carbon of X" or the carboxyl carbon of the adjacent amino acid if X" is zero;

    A' is selected from the group consisting of null (signifying no amino acid) and D- or L-cysteine;

    A" is selected from the group consisting of D- and L-cysteine;

    B' is selected from the group consisting of D- or L-histidine, D- or L-serine, D- or L-leucine, D- or L-phenylalanine, D- or L-asparagine, D- or L-proline, and D- or L-glutamine;

    C' is selected from the group consisting of D- or L-lysine, D- or L-histidine, D- or L-arginine, and D- or L-serine;

    D' is selected from the group consisting of D- or L-lysine, D- or L-leucine, D- or L-alanine, D- or L-phenylalanine, D- or L-histidine, D- or L-arginine, and D- or L-serine;

    E' is selected from the group consisting of D- or L-lysine, D- or L-phenylalanine, D- or L-glutamine, and D- or L-arginine;

    F' is selected from the group consisting of D- or L-histidine, D- or L-leucine, D- or L-alanine, D- or L-isoleucine, D- or L-threonine, and D- or L-arginine;

G' is selected from the group consisting of D- or L-alanine, D- or L-phenylalanine, D- or L-histidine, D- or L-isoleucine, and D- or L-glutamine;

H' is selected from the group consisting of D- or L-leucine, D- or L-phenylalanine, D- or L-isoleucine, D- or L-proline, and D- or L-alanine;

I' is selected from the group consisting of D- or L-cysteine, D- or L-phenylalanine, D- or L-isoleucine, D- or L-histidine, D- or L-leucine, D- or L-valine, D- or L-threonine, and D- or L- serine;

I" is selected from the group consisting of D- and L-cysteine;

J' is selected from the group consisting of D- or L-tyrosine, D- or L-phenylalanine, D- or L-isoleucine, and D- or L-valine;

R^1 is selected from the group consisting of hydrogen (signifying a free N-terminal group), lower alkyl, aryl, formyl, alkanoyl, aroyl, alkyloxycarbonyl or aryloxycarbonyl;

R^2 is selected from the group consisting of OH (signifying a free C-terminal carboxylic acid), OR^3, signifying ester, where R^3 is selected from the group consisting of lower alkyl and aryl; and NR^5R^6 where R^5 and R^6 are each selected independently from hydrogen, lower alkyl, aryl or cyclic alkyl;

and pharmaceutically acceptable salts thereof.

2.   The peptide of Claim 1 wherein R^1 is selected from the group consisting of hydrogen and acetyl.

3.   The peptide of Claim 1 wherein R^2 is selected. from the group consisting of OH and NH_2.

4.   The peptide of Claim 3 wherein R^2 is NH_2.

5.   A peptide of Claim 1 having Formula I.

6.   A peptide of Claim 5 where R^1 is selected from the group consisting of hydrogen and acetyl and R^2 is selected from the group consisting of OH and NH_2.

7.   A peptide of Claim 6 wherein R^2 is NH_2.

8.   A peptide of Claim 5 wherein, independently, A' is null; B' is selected from the group consisting of Phe, His, Leu, Asn and Ser; C' is selected from the group consisting of Lys and Arg; D' is selected from the group consisting of Lys, Phe, Leu, and Ala; E' is selected from the group consisting of Lys and Arg; F' is selected from the group consisting of Leu and Arg; G' is Ala; H' is Leu; I' is selected from the group consisting of Cys, Ile and Phe; and J' is Tyr.

9.   A peptide of Claim 8 wherein R^2 is NH_2.

10.   A peptide of Claim 1 where E' is Arg.

11.   A peptide of Claim 9 where E' is Arg.

12.   A peptide of Claim 1 having Formula II.

13.   A peptide of Claim 12 where R^1 is selected from the group consisting of hydrogen and acetyl and R^2 is selected from the group consisting of OH and NH_2.

14.   A peptide of Claim 13 wherein R^2 is NH_2.

15.   A biologically active peptide of Claim 1 selected from the group comprising:

(SEQ ID NO:1)   Cys-Leu-Lys-Lys-Lys-His-Ala-Leu-Cys-Tyr-NH_2;

(SEQ ID NO:2)   Cys-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH_2;

(SEQ ID NO:3)   Cys-His-Lys-Leu-Lys-Ala-Ala-Leu-Cys-Tyr-NH_2;

- 43 -

(SEQ ID NO:4)   cyclo-(Cys-Leu-Lys-Lys-Lys-His-Ala-Leu-Cys)-Tyr-NH$_2$;

(SEQ ID NO:5)   cyclo-(Cys-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys)-Tyr-NH$_2$;

and

(SEQ ID NO:6)   cyclo-(Cys-His-Lys-Leu-Lys-Ala-Ala-Leu-Cys)-Tyr-NH$_2$;

(SEQ ID NO:7)   Ac-Phe-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-NH$_2$;

(SEQ ID NO:8)   Phe-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-NH$_2$;

(SEQ ID NO:9)   Ac-His-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-NH$_2$;

(SEQ ID NO:10)   His-Lys-Lys-Lys-Leu-Ala-Ley-Cys-Tyr-NH$_2$;

(SEQ ID NO:11)   Leu-Lys-Lys-Lys-His-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:12)   Ac-Leu-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:13)   Leu-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:14)   Ac-Asn-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:15)   Asn-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:16)   Pro-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:17)   Gln-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:18)   Ser-His-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:19)   Ac-Ser-Lys-Ala-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:20)   Ser-Lys-Phe-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:21)   Ser-Lys-His-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:22)   Ser-Lys-Lys-Phe-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:23)   Ac-Ser-Lys-Lys-Lys-Ala-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:24)   Ser-Lys-Lys-Lys-Ala-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:25)   Ser-Lys-Lys-Lys-His-ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:26)   Ser-Lys-Lys-Lys-Ile-Ala-Leu-Cys-Tyr-NH$_2$;

- 44 -

(SEQ ID NO:27)   Ser-Lys-Lys-Lys-Leu-Ala-Phe-Cys-Tyr-NH$_2$;

(SEQ ID NO:28)   Ser-Lys-Lys-Lys-Leu-Ala-Ile-Cys-Tyr-NH$_2$;

(SEQ ID NO:29)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Phe-NH$_2$;

(SEQ ID NO:30)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Ile-NH$_2$;

5   (SEQ ID NO:31)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:32)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Phe-Tyr-NH$_2$;

(SEQ ID NO:33)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Ile-Val-NH$_2$;

(SEQ ID NO:34)   Ac-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Ile-Tyr-
                  NH$_2$;

10   (SEQ ID NO:35)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Ile-Tyr-NH$_2$;

(SEQ ID NO:36)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-His-Tyr-NH$_2$;

(SEQ ID NO:37)   Ac-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Leu-Tyr-
                  NH$_2$;

(SEQ ID NO:38)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Leu-Tyr-NH$_2$;

15   (SEQ ID NO:39)   Ac-Ser-Lys-Lys-Lys-Leu-Ala-Leu-Val-Tyr-
                  NH$_2$;

(SEQ ID NO:40)   Ser-Lys-Lys-Lys-Leu-Ala-Leu-Val-Tyr-NH$_2$;

(SEQ ID NO:41)   Ser-Lys-Lys-Lys-Leu-Ala-Ley-Thr-Tyr-NH$_2$;

(SEQ ID NO:42)   Ser-Lys-Lys-Lys-Leu-Ala-Pro-Cys-Tyr-NH$_2$;

20   (SEQ ID NO:43)   Ser-Lys-Lys-Lys-Leu-Phe-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:44)   Ser-Lys-Lys-Lys-Leu-His-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:45)   Ser-Lys-Lys-Lys-Leu-Ile-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:46)   Ser-Lys-Lys-Lys-Leu-Gln-Ala-Cys-Tyr-NH$_2$;

(SEQ ID NO:47)   Ac-Ser-Lys-Lys-Lys-thr-Ala-Leu-Cys-Tyr-
25   NH$_2$;

(SEQ ID NO:48)   Ser-Lys-Lys-Gln-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

(SEQ ID NO:49)   Ac-Ser-Lys-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-
                  NH$_2$;

(SEQ ID NO:50)   Ser-Lys-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

30   (SEQ ID NO:51)   Ac-Ser-Lys-Leu-Lys-Leu-Ala-Leu-Cys-Tyr-
                  NH$_2$;

- 45 -

    (SEQ ID NO:52)   Ser-Lys-Leu-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

    (SEQ ID NO:53)   Ac-Ser-Lys-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-
                             NH$_2$;

    (SEQ ID NO:54)   Ser-Lys-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

5   (SEQ ID NO:55)   Ac-Ser-Lys-Arg-Lys-Arg-Ala-Leu-Cys-Tyr-
                             NH$_2$;

    (SEQ ID NO:56)   Ser-Lys-Arg-Lys-Arg-Ala-Leu-Cys-Tyr-NH$_2$;

    (SEQ ID NO:57)   Ac-Ser-Lys-Arg-Arg-Leu-Ala-Leu-Cys-Tyr-
                             NH$_2$;

10   (SEQ ID NO:58)   Ser-Lys-Arg-Arg-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

    (SEQ ID NO:59)   Ser-Lys-Arg-Arg-Leu-Ala-Leu-Ser-Tyr-NH$_2$;

    (SEQ ID NO:60)   Ser-Lys-Ser-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

    (SEQ ID NO:61)   Ac-Ser-Arg-Ala-Arg-Leu-Ala-Leu-Cys-Tyr-
                             NH$_2$;

15   (SEQ ID NO:62)   Ser-Arg-Ala-Arg-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

    (SEQ ID NO:63)   Ac-Ser-Arg-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-
                             NH$_2$;

    (SEQ ID NO:64)   Ser-Arg-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

20   (SEQ ID NO:65)   Ac-Ser-Arg-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-
                             NH$_2$;

    (SEQ ID NO:66)   Ser-Arg-Lys-Arg-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

    (SEQ ID NO:67)   Ser-Arg-Lys-Arg-Leu-Ala-Leu-Ser-Tyr-NH$_2$;

    (SEQ ID NO:68)   Ac-Ser-Arg-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-
                             NH$_2$;

25   (SEQ ID NO:69)   Ser-Arg-Arg-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

    (SEQ ID NO:70)   Ser-Ser-Lys-Lys-Leu-Ala-Leu-Cys-Tyr-NH$_2$;

      16. A pharmaceutical composition comprising at
least one peptide of claim 1 in an amount effective to
inhibit cellular adherence and a pharmaceutically
30   acceptable carrier or diluent.

- 46 -

17.   A method for inhibiting leukocyte adherence in a host comprising the step of administering to said host at least one peptide of Claim 1 in an amount effective to inhibit leukocyte adherence.

5   18.   A method for modifying binding of a selectin in a host comprising administering to said host at least one peptide of Claim 1 in an amount effective to inhibit cellular adherence.

19.   The method of Claim 16 wherein said selectin is
10   selected from the group consisting of P-selectin, E-selectin and L-selectin.

20.   A method for decreasing inflammation in a host comprising administering to said host at least one peptide of claim 1 in an amount effective to decrease inflammation.

15   21.   A method for decreasing coagulation in a host comprising administering to said host at least one peptide of claim 1 in an amount effective to decrease coagulation.

22.   A method for treating a host having a condition selected from the group consisting of ischemia and
20   reperfusion, bacterial sepsis and disseminated intravascular coagulation, adult respiratory distress syndrome, tumor metastasis, rheumatoid arthritis and atherosclerosis, comprising administering to said host at least one biologically active peptide of claim 1 in an
25   amount effective to treat said condition.

23.   A method of detecting defective selectin-binding ligands and/or defective integrin-binding ligands in a host comprising the steps of:
         (a)   taking a sample of the cells to be tested from
30   said host;

(b)   contacting said cells to be tested with a labeled peptide of Claim 1; and

(c)   assessing the binding of said labeled peptide to said cell to be tested.

24.   The method of Claim 23 wherein said cells to be tested are leukocytes.

25.   A method of detecting high concentrations or elevated localized concentrations of selectin binding cells and/or integrin binding cells in a host comprising the steps of:

(a)   administering to said host a labeled peptide of Claim 1;

(b)   withdrawing a sample of cells from said host; and

(c)   assessing the binding of said labeled peptide to said sample of cells.

26.   The method of Claim 25 wherein said cells are leukocytes.

27.   The method of Claim 25 wherein said cells are tumor cells.

28.   The method of Claim 25 wherein said peptide is labeled with a moiety selected from the group comprising radioactive tracers, fluorescent tags, enzymes, and electron-dense materials.

29.   A method of preparing a peptide of Claim 1 comprising adding amino acids either singly or in pre-formed blocks of amino acids to an appropriately functionalized solid support.

30.   A method of preparing a peptide of Claim 1 comprising adding amino acids either singly or in preformed

blocks in solution or suspension by chemical ligation
techniques.

        31.   A method of preparing a peptide of Claim 1
comprising adding amino acids either singly or in preformed
blocks in solution or suspension by enzymatic ligation
techniques.

        32.   A method of preparing a peptide of Claim 1
comprising enzymatically by inserting nucleic acids
encoding the peptide into an expression vector, expressing
the DNA, and translating the DNA into the peptide.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/12110

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 1/02, 1/04, 7/06, 7/10, 7/64; C12P 21/02; A61K 37/02
US CL :514/9, 15; 530/317, 328, 334, 338, 339; 435/68.1; 930/21, 260
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/9, 15; 530/317, 328, 334, 338, 339; 435/68.1; 930/21, 260

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CA, INPADOC, JICST-E, MEDLINE search terms: selectin, fragment, peptide, ligand, binding

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US, A, 5,192,746 (Lobl et al) 09 March 1993, col. 6, line 51 - col. 7, line 12.	1-22, 29-31
A,P	US, A, 5,198,424 (McEver) 30 March 1993, col. 11, lines 2-50.	1-22, 29-31

☐ Further documents are listed in the continuation of Box C.　　☐ See patent family annex.

Date of the actual completion of the international search	Date of mailing of the international search report
14 January 1994	FEB 25 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	STEPHEN WALSH
Facsimile No.　NOT APPLICABLE	Telephone No.　(703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22, 29-31

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)⋆

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-22 and 29-31, drawn to peptides, pharmaceutical compositions, methods of treatment and methods of solid and liquid phase peptide synthesis, classified in Class 514, subclass 12.

Group II, claims 23 and 24, drawn to an in vitro method for detecting defective selectin-binding ligands, classified in Class 435, subclass 7.1.

Group III, claims 25-28, drawn to an in vivo diagnostic method, classified in Class 424, subclass 9.

Group IV, claim 32, drawn to a recombinant method of peptide synthesis, classified in Class 435, subclass 69.1.

The inventions listed as Groups I-IV do not meet the requirements for Unity of Invention for the following reasons: The Groups are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Specifically, the groups are directed to different methods practiced with materially different process steps for materially different purposes. Note that PCT Rule 13 does not provide for multiple methods within a single application.

(51) International Patent Classification 5 :		(11) International Publication Number: WO 94/19692
G01N 33/543, C12Q 1/68, C07K 15/00	A1	(43) International Publication Date: 1 September 1994 (01.09.94)

(54) **Title:** ALZHEIMER'S DISEASE THERAPEUTICS

(57) **Abstract**

A method of identifying a therapeutic useful for treating or preventing Alzheimer's disease, which method includes the steps of contacting (a) a first molecule containing the couplone portion of APP (SEQ ID NO: 1) with (b) a second molecule containing the amino acid sequence of G_o (SEQ ID NO: 2) or an APP-associating region of G_o (SEQ ID NOs: 3, 4, or 5), in the presence of a candidate compound; and determining whether the candidate compound interferes with the association of the first and second molecules, such interference being an indication that the candidate compound is a potential Alzheimer's disease therapeutic.

- 1 -

ALZHEIMER'S DISEASE THERAPEUTICS

The field of the invention is Alzheimer's disease
therapeutics.

5   Background of the Invention

Alzheimer's disease (AD) is a progressive
degenerative disorder of the brain that afflicts over
four million people in the United States. No effective
treatment is available. The most characteristic change
10 observed upon post-mortem histopathological analysis of
AD-afflicted brain tissue is the presence of neuritic and
cerebrovascular plaques containing dense deposits of β-
amyloid protein (Selkoe, Cell 58:611-612, 1989). β-
amyloid is a 39-43 amino acid peptide (Glenner and Wong,
15 biochem. biophys. Res. Commun. 120:885-890, 1984; Masters
et al., Proc. Natl. Acad. Aci. USA 82:4345-4249, 1985)
synthesized as part of a larger precursor protein
referred to as amyloid precursor protein (APP), which is
known to have a number of isoforms in humans (APP_{695}, Kang
20 et al., Nature 325:733-736, 1987; APP_{751}, Ponte et al.,
Nature 331:525-527, 1988, and Tanzi et al., Nature
331:528-530, 1988; and APP_{770}, Kitaguchi et al., Nature
331:530-532, 1988). The amino terminal of β-amyloid is
generated by cleavage of a peptide bond of APP which in
25 APP_{695} lies between Met596 and Asp597.

Although structural alterations of APP are
implicated in the pathogenesis of Alzheimer's disease, it
remains unknown how they cause the disease. No
biological function for APP has been identified, although
30 there is evidence that APP has a receptor-like
architecture (Kang et al., Nature 325:733-736, 1987;
Ponte et al., Nature 331:525-527, 1988; Tanzi et al.,
Nature 331:528-530, 1988; Kitaguchi et al., Nature
331:530-532, 1988), is located on the neuronal surface
35 (Dyrks et al., EMBO J. 7:949-957, 1988), and possesses an

- 2 -

evolutionarily conserved cytoplasmic domain (Yamada et
al., Biochem. Biophys. Res. Commun. 149:665-671, 1987).

Summary of the Invention

The methods and therapeutical compositions of the
5   invention are based upon the discovery, described in
detail below, that APP forms a complex with G_o, a major
GTP-binding protein (or "G protein") in brain. Like all
G proteins, a molecule of G_o is made up of one α subunit
and one $\beta\gamma$ subunit. Two isoforms of G_o, known as G_{o1} (or
10 G_{oA}) and G_{o2} (or G_{oB}), have been identified; they have
slight amino acid differences in their α subunits, and
are together referred to herein as G_o. The cDNA sequence
and deduced amino acid sequence of the α subunits of each
of G_{o1} and G_{o2} (as reported by Strathmann et al., Proc.
15 Natl. Acad. Sci. USA 87:6477-6481, 1990) are shown in
Fig. 4a (SEQ ID NO: 2) and Fig. 4b (SEQ ID NO: 28),
respectively.

The finding that APP associates with G_o is
consistent with related findings concerning other
20 G proteins, as disclosed in a second application
(USSN_____) having the same inventor and filing
date as the present application, which second application
is herein incorporated by reference. The cytoplasmic
APP_{695} sequence His^{657}-Lys^{676} (SEQ ID NO: 1) possesses a
25 specific G_o-activating function, and is necessary for
complex formation of this APP with G_o; this sequence,
sometimes referred to as the "couplone" region of APP, is
completely conserved in APP_{751} and APP_{770}, as well as in
mouse APP_{695}. This provides evidence that APP is a
30 receptor coupled to G_o, and suggests that abnormal APP-G_o
signalling is involved in the Alzheimer's disease
process.

- 3 -

The invention includes a method of identifying a
therapeutic useful for treating or preventing Alzheimer's
disease, which method includes the steps of
contacting (a) a first molecule containing the
5   couplone portion of APP (SEQ ID NO: 1) with (b) a second
molecule containing the amino acid sequence of G_O (SEQ ID
NO: 2) or an APP-associating region of G_O (SEQ ID NOs: 3,
4, or 5), in the presence of a candidate compound; and
either (i) determining whether the candidate
10   compound interferes with (i.e., inhibits partially or
completely) the association of the first and second
molecules, or (ii) determining whether the candidate
compound interferes with the activation of the second
molecule by the first molecule, such interference being
15   an indication that the candidate compound is a potential
therapeutic useful for treating or preventing Alzheimer's
disease. The determining step may be accomplished by,
for example, immunoprecipitating the first molecule with
an antibody specific for APP, and detecting the presence
20   or amount of the second molecule which co-precipitates
with the first molecule. Alternatively, the second
molecule can be immunoprecipitated with an antibody
specific for G_O, following which the presence or amount
of the first molecule which co-precipitates with the
25   second molecule is determined. Where activation is the
criterion being measured, the determination step may be
accomplished by contacting the second molecule with a
substrate which is or includes GTP or an analog of GTP
[such as GTPγS or Gpp(NH)p], and detecting or measuring
30   the binding of the substrate to the second molecule,
wherein such binding is evidence of activation of the
second molecule by the first molecule. In preferred
embodiments, the contacting step is carried out in a
cell-free system; the Mg^{2+} concentration at which the
35   contacting step is carried out is between approximately

- 4 -

1×10^{-7} and 1×10^{-2} M, and the first molecule includes the
cytoplasmic tail portion of APP_{695} from residues 649 to
695 (SEQ ID NO: 6) and/or the membrane-spanning portion
of APP_{695} from residues 639 to 648 (SEQ ID NO: 7) (the

5   entire membrane-spanning segment of APP_{695} being from
residues 625 to 648, SEQ ID NO: 8); the first molecule
more preferably includes substantially all of APP (SEQ ID
NO: 9). (Alternatively, the corresponding functional
regions of APP_{751} or APP_{770}, or any other APP, may be

10   used.) The second molecule preferably contains two or
three of the putative APP-associating regions referred to
above, and may also contain one or more of the GTP-
binding regions of G_o, corresponding to residues 35 to 50
(SEQ ID NO: 10), residues 201 to 218 (SEQ ID NO: 29), or

15   residues 263 to 274 (SEQ ID NO: 30) of G_{o1} [Kaziro,
"Structure of the genes coding for the α subunits of G
proteins", Ch. 1 in ADP-ribosylating Toxins and G
proteins (Moss, J., and Vaughan, M. eds.) pp189-206,
American society for Microbiology, Washington, D.C.

20   (1988)], and more preferably contains substantially all
of G_o (SEQ ID NO: 2).

The invention also includes a system (e.g., a
cell-free in vitro system) for screening candidate
Alzheimer's disease therapeutics, which system includes a

25   first polypeptide containing a sequence essentially
identical to that of peptide 20 (SEQ ID NO: 1), and a
second polypeptide containing a sequence essentially
identical to one, two or three of the putative APP-
associating regions of G_o (SEQ ID NOs: 3, 4, and 5); the

30   system may also include a means for detecting either (a)
the association of the first polypeptide with the second
polypeptide, or (b) the activation of the second
polypeptide by the first polypeptide. The first
polypeptide may conveniently be anchored to a solid

35   material (e.g., a cellular membrane, a polystyrene

- 5 -

surface, or a standard matrix material), or may be in a
phospholipid vesicle. It may include a sequence
essentially identical to the membrane-spanning region of
APP, and/or a sequence essentially identical to the
5   entire cytoplasmic tail of APP. The second molecule
preferably contains the GTP-binding domain of G_O, and
more preferably contains the entire sequence of G_O.

The invention also features a method for
diminishing the activation of G_O in a neuronal cell by
10   treating the cell with a compound, such as a peptide
fragment of G_O or of the cytoplasmic tail of APP, which
blocks association of neuronal G_O with, and/or activation
of neuronal G_O by, the cytoplasmic tail of APP. The cell
may be so treated in vivo (i.e., in an animal, e.g. a
15   mammal such as a human or other primate, cow, horse, pig,
sheep, goat, dog, cat, rat, mouse, guinea pig, hamster,
or rabbit) or in vitro. This method may be used to
prevent or treat the symptoms of Alzheimer's disease in a
patient. Such a compound may include, for example, a
20   peptide having fewer than 50 amino acids (preferably 40
or fewer, and more preferably 30 or fewer), and
containing the sequence of peptide 20. Also within the
invention is a DNA molecule (e.g., a plasmid or viral
DNA) encoding such a peptide, and a therapeutic
25   composition containing, in a pharmaceutically acceptable
carrier, either the peptide or the DNA molecule.

In another aspect, the invention features a method
for identifying a ligand for which APP is a receptor,
which method includes the steps of
30   providing an APP molecule, the cytoplasmic tail of
which is accessible to a molecule of G_O;
contacting a candidate compound with the
extracellular domain of the APP molecule; and
detecting either (a) association of G_O with the
35   APP molecule, (b) dissociation of G_O from the APP

- 6 -

molecule, or (c) activation of G_o by the APP molecule, such association, dissociation, or activation being evidence that the candidate compound is a ligand of APP.

Other features and advantages of the invention will be apparent from the detailed description set forth below, and from the claims.

Brief Description of the Drawings

Fig. 1(a) is a schematic diagram illustrating the structural organization of APP. The hatched box contains the sequence of the β/A_4 protein; the black box contains the so-called "Peptide 20" or couplone sequence; filled circles are N-glycosylation sites. The numbers designate amino acid sequence numbers corresponding to APP_{695}.

Fig. 1(b) is a bar graph illustrating the effects of synthetic APP peptides on G_o. In (b), (d), (e) and (f), values represent the mean ±S.E. of three experiments.

Fig. 1(c) is a graph illustrating the time course of the action of peptide 20 on G_o. Values represent the mean of three experiments. Since the S.E. was < 5% of each value in this figure, the error bars are not indicated.

Fig. 1(d) is a graph illustrating the effects of peptide 20 variants on G_o.

Fig. 1(e) is a graph illustrating the effect linkage with a transmembrane region has on the action of peptide 20 on G_o.

Fig. 1(f) is a graph illustrating the effect of pertussis toxin on peptide 20-induced stimulation of GTP-γS binding to G_o.

Figs. 2a-2d is a set of SDS-PAGE gels analyzed by immunoblotting, which illustrate the immunoprecipitation of APP and G_o by an anti-APP antibody from brain membranes. (a) Immunoprecipitation of APP by 22C11.

- 7 -

(b) Immunoprecipitation of G_o by 22C11.   (c) Effect of Mg^{2+} on the immunoprecipitation of G_o by 22C11.

(d) Effect of peptide 20 on 22C11-induced precipitation of $G_{o\alpha}$ (*left*) and APP (*right*).   Each of the results

5 presented in this figure was reproduced at least three times.

Fig. 3a is a schematic diagram of the construction method used to prepare recombinant mutant APP cDNAs. Regions labeled ATG, TAA, TGA signify original

10 translation and termination sites and a newly inserted termination site, respectively.

Fig. 3b is a schematic diagram comparing the structures of authentic APP_{695} and the two recombinant mutant APP polypeptides, ΔN and ΔC.

15   Fig. 3c is an immunoblot analysis of Sf9 membranes using anti-Alz 90, 1C1, and 4G5.

Fig. 3d is an immunoblot analysis of the 22C11-precipitate from an Sf9 membrane-G_o reconstitution mixture.

20   Fig. 3e is an immunoblot illustrating dissociation of G_o from APP by activation of G_o.   Each of the results presented in Figs. 3c-e was reproduced at least three times.

Fig. 4a is the cDNA sequence and deduced amino

25 acid sequence of $G_{o1}\alpha$ (Strathmann et al., Proc. Natl. Acad. Sci. USA 87:6477-6481, 1990) (SEQ ID NO: 2).

Fig. 4b is the cDNA sequence and deduced amino acid sequence of $G_{o2}\alpha$ (Strathmann et al.) (SEQ ID NO: 28).

Detailed Description

30   It was previously shown that the insulin-like growth factor II receptor (IGF-IIR) couples directly to the G protein referred to as G_i (Nishimoto et al., J. Biol. Chem. 264:14029-14038, 1989) via a 14-residue section of the cytoplasmic tail of IGF-IIR, $Arg^{2410}-Lys^{2423}$

- 8 -

(Okamoto et al., Cell 62:709-717, 1990; Okamoto et al.,
Proc. Natl. Acad. Sci. U.S.A. 88:8020-8023, 1991). The
structural determinants for the G_i-activating function in
IGF-IIR were defined as (i) two basic residues at the N-
5   terminal region of the amino acid sequence, and (ii) a C-
terminal motif of *B-B-X-B* or *B-B-X-X-B* (where *B* is a
basic residue and *X* is a non-basic residue) (Okamoto et
al., Cell 62:709-717, 1990). To assess whether APP might
function as a G protein-coupled receptor, the amino acid
10 sequence of human APP695 was examined for regions of less
than 26 residues which satisfy (i) and (ii). The
sequence His[657]-Lys[676] is the only such region in the
cytoplasmic domain of APP695. In two other isoforms of
APP, APP751 (Ponte et al., Nature 331:525-527, 1988; Tanzi
15 et al., Nature 331:528-530, 1988) and APP770 (Kitaguchi et
al., Nature 331:530-532, 1988), as well as in mouse APP695
(Yamada et al., Biochem. Biophys. Res. Commun. 149:665-
671, 1987), this sequence is completely conserved.

Preparation of peptides
20   A peptide corresponding to the His[657]-Lys[676] region
of APP [HHGVVEVDAAVTPEERHLSK (SEQ ID NO: 1)] was
synthesized and purified by standard methods using solid
phase synthesis; this peptide is referred to as
"peptide 20". Similarly prepared were peptides
25 corresponding to other regions of APP$_{695}$:
APP(1-10), MLPGLALLLL (SEQ ID NO: 11);
APP(597-606), DAEFRHDSGY (SEQ ID NO: 12);
APP(677-695), MQQNGYENPTYKFFEQMQN (SEQ ID NO: 13);
and APP(639-648), TVIVITLVML (SEQ ID NO: 7), a portion of
30 the transmembrane region of APP;
as well as the following variants of peptide 20:
HGVVEVDAAVTPEERHLSK (H-deleted, SEQ ID NO: 14);
GVVEVDAAVTPEERHLSK (HH-deleted, SEQ ID NO: 15);
HHGVVEVDAAVTPEE (RHLSK-deleted, SEQ ID NO: 16);

- 9 -

KQYTSIHHGVVEVDAAVTPEERHLSK (KQYTSI-added, SEQ ID NO: 17);
and <u>TVIVITLVML</u>HHGVVEVDAAVTPEERHLSK (transmembrane region-
connected peptide 20; SEQ ID NO: 18).

Peptides were purified by HPLC to greater than 95%
5 purity, and were used immediately after synthesis.

Materials and Methods.

Trimeric G_o was purified to homogeneity from
bovine brain as described (Katada et al., FEBS Lett.
213:353-358, 1987). This G_o preparation was stored in 20
10 mM Hepes/NaOH (pH 7.4), 1 mM EDTA, and 0.7% CHAPS, and
diluted ≥ 10 fold for assays. $G_{i3\alpha}$, which was used in
combination with 1.5-fold concentrated $G\beta\gamma$ (Okamoto et
al., Natl. Acad. Sci. U.S.A. 88:8020-8023, 1991), was
prepared as described by Morishita et al., Biochim.
15 Biophys. Acta 161:1280-1285, 1989. Low molecular weight
G proteins were prepared as described by Matsui et al.,
J. Biol. Chem. 263:11071-4, 1988; $G\beta\gamma$ was purified from
bovine brain as set forth in Katada et al., FEBS Lett.
213:353-358, 1987.

20   GTPγS binding to G_o was assayed in a buffer
containing 50 mM Hepes/NaOH (pH 7.4), 100 μM EDTA, 120 μM
$MgCl_2$, and 60 nM [^{35}S]GTPγS (DuPont-New England Nuclear)
at 37°C, and the fraction of total G_o bound to GTPγS was
measured as described (Okamoto et al., Cell 62:709-717,
25 1990). GTPγS binding to peptides was negligible. The
total amount of G_o in a given preparation was defined as
the saturation amount of GTPγS bound to G_o following a
30-min incubation of G_o with 10 mM Mg^{2+} and ≥ 60 nM GTPγS
at 30°C.

30   Reconstitution of G_o into phospholipid vesicles
was accomplished with 1 mg/ml of phosphatidylcholine,
using the gel filtration method (Nishimoto et al., J.
Biol. Chem. 264:14029-14038, 1989). In a final

- 10 -

incubation for GTPγS binding, 5 nM of reconstituted G_o was used.

For experiments exploring the effect of Mg^{2+}, the Mg^{2+} concentration was set by using Mg-EDTA buffer

5   (Birnbaumer et al., J. Eur. J. Biochem. 136:107-112, 1983).

Bovine brain membranes, prepared as described (Katada et al., FEBS Lett. 213:353-358, 1987) and suspended in buffer A [10 mM Hepes/NaOH (pH 7.4), 1 mM

10 EDTA, 10 mM acetic acid, and 250 mM sucrose, plus a mixture (termed "PAL") of 2 mM PMSF, 20 μg/ml aprotinin, and 20 μM leupeptin], were centrifuged and the pellet was solubilized for 1 h at 4°C in buffer B (10 mM Hepes/NaOH (ph 7.4), 1 mM EDTA, 120 mM NaCl, 0.5% CHAPS, and PAL).

15 Following centrifugation of the material at 15000 rpm for 1 h, the supernatant (500 μg protein, unless specified) was incubated in buffer C (20 mM Hepes/NaOH (pH 7.4), 1 mM EDTA, 120 mM NaCl, and PAL) and 2% BSA with 22C11-coated protein G-Sepharose, which had been prepared by

20 incubating protein G-Sepharose (Pharmacia) with anti-APP monoclonal antibody 22C11 (Boehringer Mannheim) for 1 h at 4°C. An antibody concentration of ≥ 2 μg/ml was found to saturate precipitation of APP and G_o, so 2 μg/ml was the concentration used for immunoprecipitation studies.

25 As a control, 2 μg/ml of rabbit IgG was used. After overnight shaking at 4°C, the immunoprecipitated sample was centrifuged at 5000 rpm for 5 min. The pellet was washed three times with ice-cold buffer C and the final pellet was applied to SDS-PAGE. Electroblotting onto a

30 PVDF sheet was performed as described (Okamoto et al., J. Biol. Chem. 266:1085-1091, 1991). After blocking with PBS containing 2% skim milk and 1% BSA, the sheet was incubated with the first antibody [1 μg/ml of 22C11; 1/1000 dilution of anti-$G_o\alpha$ monoclonal antibody GC/2

35 (DuPont-New England Nuclear); 1/1000 dilution of 1C1, a

- 11 -

monoclonal antibody against the C-terminal peptide $_{677-695}$
of APP$_{695}$] for 4 h, and then exposed to horseradish
peroxidase-conjugated goat IgG reactive for mouse or
rabbit immunoglobulins for 2-4 h at room temperature.
5   The antigenic bands were detected with an ECL detection
kit (Amersham). YL1/2 (SERA Lab), an anti-tubulin
antibody, was used at 1:500 dilution for immunodetection.

Effects of synthetic APP peptides on G proteins.

         In the experiment shown in Fig. 1(b), 10 nM G$_O$ was
10 incubated with water or 100 μM of each peptide for 2 min,
and the amount of GTPγS bound to G$_O$ at the end of this
period was measured. In the experiment shown in Fig.
1(c), 10nM G$_O$ was incubated with water (O) or 100 μM
peptide 20 (SEQ ID NO: 1) (●), and GTPγS binding was
15 measured at the indicated times. From Fig. 1(d), it can
be seen that peptide 20 (SEQ ID NO: 1) stimulated the
rate constant of GTPγS binding to G$_O$ in a dose-dependent
manner, whereas Fig. 1(b) shows that peptides from other
regions of APP695 were ineffective. GTPγS binding to G$_O$
20 in the presence or absence of peptide 20 (SEQ ID NO: 1)
obeyed first-order kinetics according to the equation
         ln [*(BT-B)/BT*]=-$k_{app}t$
(*B* is the binding at time *t*; *BT* is the total binding
observable at infinite time; and k_{app} is the rate constant
25 for GTPγS binding). The ability of peptide 20 (SEQ ID
NO: 1) to activate G$_O$ was gradually decreased during
storage at either -4°C or -20°C.
         Studies using structural variant peptides suggest
that both the N-terminal basic residues and the C-
30 terminal *B-B-X-X-B* motif play essential roles in the G$_O$-
activating function of peptide 20 (SEQ ID NO: 1) [Fig.
1(d)]. In this experiment, 10 nM G$_O$ was incubated with
various concentrations of HHGVVEVDAAVTPEERHLSK (peptide
20, SEQ ID NO: 1; □), HGVVEVDAAVTPEERHLSK (H-deleted, SEQ

- 12 -

ID NO: 14; ◊), GVVEVDAAVTPEERHLSK (HH-deleted, SEQ ID
NO: 15; □), HHGVVEVDAAVTPEE (RHLSK-deleted, SEQ ID
NO: 16; ♦), or KQYTSIHHGVVEVDAAVTPEERHLSK (KQYTSI-added,
SEQ ID NO: 17; ■), and GTPγS binding to G_O at 2 min. was
5   measured. Fig. 1(d) indicates which aspects of primary
structure determine the G_O-activator function of peptide
20 (SEQ ID NO: 1). Deletion of either one or both of the
N-terminal His residues nullified G_O-activator function
of the peptide. The peptide (SEQ ID NO: 16) in which the
10   C-terminal five residues of peptide 20 (SEQ ID NO: 1) has
been deleted is several times less potent than peptide 20
(SEQ ID NO: 1).

As illustrated in Fig. 1(e), G_O reconstituted in
phospholipid vesicles was incubated with transmembrane
15   region-connected peptide 20
(TVIVITLVMLHHGVVEVDAAVTPEERHLSK, SEQ ID NO: 18; □) or the
partial sequence of the APP transmembrane domain alone
(TVIVITLVML, SEQ ID NO: 7; □). Transmembrane region-
connected peptide 20 (SEQ ID NO: 18) was also incubated
20   with G_O in the absence of phospholipids and the presence
of 0.07% CHAPS (♦). The transmembrane region-connected
peptide 20 (SEQ ID NO: 18) stimulated G_O reconstituted in
phospholipid vesicles with a potency 10 times greater
than that of peptide 20 (SEQ ID NO: 1). The
25   transmembrane region alone (SEQ ID NO: 7) was without
effect on G_O. In the absence of phospholipids,
transmembrane region-connected peptide 20 (SEQ ID NO: 18)
showed an effect on G_O no more potent than peptide 20
(SEQ ID NO: 1). Therefore, the stimulatory action of
30   this transmembrane region-connected peptide (SEQ ID
NO: 18) is attributed to the peptide 20 (SEQ ID NO: 1)
sequence; the potentiating effect of the transmembrane
region may be exerted by interactions with phospholipids.

In the experiment shown in Fig. 1(f), ADP-
35   ribosylation of G_O was accomplished by incubating G_O

- 13 -

reconstituted in phospholipid vesicles with 10 μg/ml preactivated pertussis toxin in the presence of 10μM NAD for 15 min at 30°C as described (Okamoto et al,, Cell 62:709-717, 1990). Preactivation of pertussis toxin

5   (Funakoshi, Japan) was carried out by treating the toxin with 100 μM ATP and 1 mM DTT for 10 min at 30°C. Reconstitution of G_o into phospholipid vesicles was accomplished with 1 mg/ml phosphatidylcholine (Sigman, P-5638) at a final G_o concentration of 50.2 nM in a buffer

10 containing 20 mM Hepes/NaOH (pH 7.4), 0.1 mM EDTA, 1 mM DTT, and 100 mM NaCl by the gel filtration method (Nishimoto et al., J. Biol. Chem. 264:14029-14038, 1989). In a final incubation for GTPγS binding, 5 nM of reconstituted G_o was used. Increasing concentrations of

15 peptide 20 (SEQ ID NO: 1) were incubated for 2 min with G_o reconstituted in phospholipid vesicles which had been treated with pertussis toxin in the presence (♦) or absence (□) of NAD, and GTPγS binding to G_o was measured.

        Although peptide 20 (SEQ ID NO: 1) produced 2-3
20 fold stimulation of GTPγS binding to G_o in the mid-range of Mg^{2+} concentrations, the effect of peptide 20 (SEQ ID NO: 1) could not be observed at low ($\leq$ 100 nM) or high ($\geq$ 10 mM) Mg^{2+} concentrations.

        Peptide 20 (SEQ ID NO: 1) had little effect on G
25 proteins other than G_o: G_{i1}, G_{i2}, G_{i3}, G_s, c-Ki-*ras* p21 and *smg* p25A were not stimulated by this peptide (data not shown). Thus, peptide 20 (SEQ ID NO: 1) activates G_o in a receptor-like manner, suggesting that APP interacts directly with G_o through the peptide 20 (SEQ ID NO: 1)
30 region.

Coprecipitation of APP and G_o

        In an effort to determine whether APP is linked to G_o in a native membrane environment, the coprecipitation studies shown in Fig. 2a were performed. Solubilized
35 membranes of bovine brain were first immunoprecipitated

- 14 -

by monoclonal anti-APP antibody 22C11, and the
immunoprecipitate was then probed by immunodetection with
22C11 (Lane 2) or 1C1, a monoclonal antibody against the
C-terminal peptide$_{677-695}$ of APP (SEQ ID NO: 13; Lane 4).

5   Lanes 1 and 3 of Fig. 2a indicate the controls in which
either no solubilized membranes were included (Lane 1),
or rabbit IgG was used for the precipitation step instead
of antibody 22C11 (Lane 3). In each control,
immunodetection was performed with 22C11. The 55-kDa and

10 25-kDa bands seen in Lanes 1 and 2 may be heavy and light
chains of the 22C11 used for precipitation, which reacted
with an anti-mouse IgG antibody during immunodetection.
The precipitate by control rabbit IgG contained no
detectable APP. Although the 100 kD molecular size of

15 APP appears here to be slightly less than the 110-130 kD
reported (Weidemann et al., Cell 57:115-126, 1989), the
precipitated form is unlikely to be an extracellular
fragment of APP, because 1C1 recognizes this 100-kDa
band.

20   In the experiment illustrated in Fig. 2b,
coprecipitation of various G proteins with APP was
investigated. Bovine brain membrane preparations were
immunoprecipitated with 22C11; the immunoprecipitated
proteins were subjected to SDS-PAGE and immunoblotted

25 with the indicated anti-G protein antisera (1/1000
dilution). Lane 2: GC/2, anti-G$_o\alpha$ antiserum; lane 3:
GC/2 plus 1 μg/ml of purified G$_o$; lane 4: GA/1, common Gα
antiserum; lane 5: AS/7, anti-Giα antiserum; lane 6:
MS/1, common Gβ antiserum. Lane 1 shows a control

30 immunoblot with GC/2, in which a buffer solution rather
than the bovine brain membrane preparation was
immunoprecipitated with 22C11. Lane 7 indicates
immunoblotting with GC/2 of the precipitate resulting
from immunoprecipitation of brain membranes with control

35 rabbit IgG, rather than 22C11. The identity of the 39-

- 15 -

kDa protein in lane 2 as G_o was verified by its absence
in the non-membrane control (lane 1); by its staining
with another $G_o\alpha$-specific antibody, $\alpha GO1$ (Morishita et
al., Eur. J. Biochem. 174:7-94, 1988) (data not shown);
5 and by a diminution of staining of this band in the
presence of excess soluble G_o (lane 3). The 22C11-
precipitate also contained immunoreactivity of $G\beta$ in a
doublet at 35-36-kDa (lane 6). The 22C11-precipitate did
not react with an anti-$Gi\alpha$ antibody AS/7 (lane 5). The
10 antibody GA/1 detected only a 39-kDa band in the 22C11-
precipitate (lane 4). The control rabbit IgG
immunoprecipitate did not produce anti-G_o-immunoreactive
bands corresponding to either APP or G_o (lane 7). These
experiments indicate that the 22C11-precipitate from
15 brain membranes contains APP immunoreactivity at 100 kDa,
$G_o\alpha$ immunoreactivity at 39 kDa, and $G\beta$ immunoreactivity
in a doublet at 35-36 kDa, but no detectable
immunoreactivity indicating the presence of $G_i\alpha$ or other
heterotrimeric G proteins. A tubulin antibody, YL1/2,
20 did not stain the 22C11-precipitate (data not shown).

        In the experiment shown in Fig. 2c, the effect
of Mg^{2+} concentration on co-precipitation of G_o with anti-
APP antibody was studied. 100 μg of solubilized brain
membranes were precipitated by 22C11 in the presence of
25 various Mg^{2+} concentrations controlled with Mg-EDTA buffer
(Birnbaumer et al., J. Eur. J. Biochem. 136:107-112,
1983). The precipitates were analyzed by immunoblotting
with GC/2. The control lane indicates the results of
precipitation of brain membranes by rabbit IgG followed
30 by immunodetection with GC/2. In the absence of Mg^{2+}, G_o
was less efficiently co-precipitated by 22C11. Mg^{2+}
concentrations between 1 μM and 1 mM resulted in maximal
immunoprecipitation of G_o. At concentrations > 10 mM,
relatively little G_o was precipitated. In contrast,
35 immunoprecipitation of APP by 22C11 was not affected by

- 16 -

Mg^{2+} concentration (data not shown). These results
indicate that, while Mg^{2+} is not absolutely required for
complex formation by APP and G_o, the concentration of Mg^{2+}
does strongly influence complex formation. A mid range
5 of Mg^{2+} concentration was found to facilitate APP-G_o
association.

        Fig. 2d illustrates the results of an experiment
indicating that peptide 20 (SEQ ID NO: 1) prevents the
22C11-mediated co-precipitation of G_o, whereas it did not
10 affect the precipitation of APP by 22C11. In contrast, a
control peptide (SEQ ID NO: 13) representing a segment of
APP different from that represented by peptide 20 (SEQ ID
NO: 1) had no discernable effect on 22C11-mediated co-
precipitation of G_o. In this experiment, solubilized
15 brain membranes were incubated with 22C11-coated beads in
the presence of 10 μM peptide 20 (SEQ ID NO: 1; 2nd and
5th lanes) or 10 μM of the control peptide, peptide$_{677-695}$
of APP (SEQ ID NO: 13; 3rd and 6th lanes), or in the
absence of both of these peptides (1st and 4th lanes).
20 In this experiment, an anti-mouse IgG antibody different
from that used in (a) was employed.

**Precipitation of G_o reconstituted with recombinant APP-
antibody complex**

        A baculovirus DNA encoding full-length APP$_{695}$ (SEQ
25 ID NO: 9) was prepared as outlined in Fig. 3a. Authentic
mouse APP$_{695}$ cDNA (SEQ ID NO: 9) was provided by Dr.
Yoshiyuki Sakaki (University of Tokyo, Japan) (Yamada et
al., Biochem. Biophys. Res. Commun. 149:665-671, 1987) in
the vector pUC18. The HindIII-BamHI fragment containing
30 the entire coding region was initially subcloned into the
vector pBR322 (pBR-APP). A single BamHI site was
inserted immediately before the ATG codon of the HindIII-
SphI fragment. This BamHI site was inserted to permit
efficient expression of the encoded APP protein in

- 17 -

baculovirus-infected cells. The BamHI site-inserted
APP$_{695}$-coding DNA (BamHI-APP$_{695}$) was constructed from the
HindIII-SphI fragment and pBR-APP, utilizing their
internal KpnI sites, and subcloned into pUC18. By using
5   BamHI-APP$_{695}$ as template, two truncation mutants were
generated and subcloned into pUC18. These mutants
possess an insertion of two TGA codons immediately before
(ΔN) or after (ΔC) the peptide 20 sequence. Each BamHI-
BamHI fragment of these respective APP-variation-encoding
10 pUC18 plasmids was inserted into the baculovirus
transfer/expression vector pVL1393 (Invitrogen). The
entire region that had been through a single-stranded
intermediate was sequenced to confirm the absence of
unwanted nucleotide changes. New insertions were
15 generated by oligonucleotide-directed mutagenesis with a
kit (Takara) by the method of Kunkel et al. (Meth.
Enzymol. 154:367-382, 1987). For the insertion of a
BamHI site, a restriction fragment encoding the ATG start
codon was subcloned into the vector M13mp18 and a single
20 stranded template was generated. An oligonucleotide
primer (CCACGCAGGATCACGGGATCCATGCTGCCCAGCTTG; SEQ ID
NO: 19) was used to introduce GGATCC (SEQ ID NO: 20)
immediately before the start codon. Following primer
extension, the phage was used to transform E. coli strain
25 JM109. Plaques were selected and single stranded DNA was
sequenced. A restriction fragment containing the mutated
region was subcloned into pBR-APP. For the insertion of
the stop codons, oligonucleotide primers
[CAGTACACATCCATCTGATGACATCATGGCGTGGTG (SEQ ID NO: 21) and
30 CGCCATCTCTCCAGTGATGAATGCAGCAGAACGGA (SEQ ID NO: 22)] and
the M13mp19 vector were used to introduce two sequential
TGA stop codons. Using the method of Summers and Smith
(Summers et al., Tex. Agric. Exp. Stn. Bull. 1555, 1987),
baculoviruses incorporating these APP cDNAs were
35 generated using selection by immunoblot analysis with

- 18 -

22C11, and recovered by infecting Sf9 cells (Invitrogen).
Four days after treatment of Sf9 cells with the viruses,
cells were homogenized and suspended in buffer A. After
the solubilization of the pellet with buffer B, the

5 supernatant (100 μg) was mixed overnight with 22C11-
coated protein G-Sepharose in buffer C plus 2% BSA at 4°C
on a shaker. After centrifugation, the precipitated
beads were incubated with purified G_o (1 μg) in buffer C
supplemented with 1.1 mM MgCl2 and 2% BSA for 8-24 h at

10 4°C on a shaker. After washing four times with ice-cold
buffer C, the centrifugation precipitate was subjected to
SDS-PAGE, electroblotting, and immunodetection with the
first antibodies (1 μg/ml of 22C11; 10 μg/ml of anti-Alz
90; 1/1000 dilution of 1C1; 1/500 dilution of 4G5; 0.1

15 μg/ml of αGO1) and the second goat anti-mouse or anti-
rabbit IgGs conjugated with HRP. (Immunodetection of 1C1
and 4G5, both of which are mouse IgM (κ), was
accomplished using as second antibody a mixture of HRP-
conjugated anti-rabbit IgG, rabbit anti-mouse IgM and

20 rabbit anti-mouse κ antibodies.)   The
three APP constructs prepared as described above are
compared in the schematic diagram of Fig. 3b. The
polypeptides encoded by all three constructs retain the
entire transmembrane and extracellular domains of APP;

25 while ΔN (SEQ ID NO: 23) lacks all of the peptide 20
residues as well as the sequence on the carboxy terminal
side of the peptide 20 region, ΔC (SEQ ID NO: 24) retains
the peptide 20 sequence and is missing only the latter
sequence.

30   Sf9 cells were infected, using standard methods,
by recombinant baculoviruses encoding full length APP_{695}
cDNA (SEQ ID NO: 9), APP_{1-656} cDNA (ΔN; SEQ ID NO: 23), or
APP_{1-676} cDNA (ΔC; SEQ ID NO: 24). In uninfected Sf9
cells, no immunoreactivity for anti-APP or anti-G_o

35 antibodies was detected (data not shown). The membranes

- 19 -

of Sf9 cells infected with the baculoviruses encoding
APP$_{695}$ (SEQ ID NO: 9), ΔN (SEQ ID NO: 23), and ΔC (SEQ ID
NO: 24) genes (referred to as Sf9-APP$_{695}$, Sf9-ΔN, and Sf9-
ΔC, respectively) were found to express, respectively,
5   130-, 120- and 130-kDa proteins reactive with antibody
22C11 (Fig. 3d, right side). The Sf9-APP$_{695}$ cells
expressed APP at ≈ 0.1% of the total membrane protein.
When the membranes of the three types of infected cells
were immunoprecipitated with antibody Anti-Alz 90
10   (Boehringer Mannheim), a mouse monoclonal antibody
specific for an epitope corresponding to to residues 551-
608 of APP (SEQ ID NO: 25; a section of APP that is
within the extracellular domain), 130-kDa, 120-kDa, and
130-kDa proteins were recognized in Sf9-APP$_{695}$, Sf9-ΔN,
15   and Sf9-ΔC cells, respectively (Fig. 3c, top panel).
Membranes from all three types of infected cells showed
approximately equivalent reactivity to the antibody,
indicating that at least this portion of the
extracellular domain was intact on each of the three and
20   that all three cell types express approximately equal
amounts of recombinant protein. When the antibody used
was 1C1, a mouse monoclonal prepared against a peptide
corresponding to residues 677-695 of APP (SEQ ID NO: 13),
only Sf9-APP$_{695}$ membranes were reactive, indicating that
25   the region corresponding to the C-terminal portion of the
cytoplasmic domain is missing from both ΔN (SEQ ID
NO: 23) and ΔC (SEQ ID NO: 24) (Fig. 3c, middle panel).
When the antibody used was 4G5, a mouse monoclonal
antibody raised against a peptide corresponding to
30   residues 657-676 of APP (SEQ ID NO: 1; the peptide 20
region of the cytoplasmic domain), 130 kDa bands from
both Sf9-APP$_{695}$ and Sf9-ΔC membranes reacted with the
antibody, but Sf9-ΔN membranes did not, a demonstration
that ΔN (SEQ ID NO: 23) but not ΔC (SEQ ID NO: 24) lacks
35   the peptide 20 region of APP (Fig. 3c, bottom panel).

- 20 -

These experiments clearly indicate that the expressed
proteins are recombinant APP_{1-695} (SEQ ID NO: 9), APP_{1-656}
(SEQ ID NO: 23), and APP_{1-676} (SEQ ID NO: 24),
respectively, as designed.

5   The 22C11-precipitates from these Sf9 membranes
expressing various forms of APP were exposed to purified
G_o, reprecipitated with 22C11, and subjected to
immunoblot analysis using anti-$G_o\alpha$ antibody $\alpha GO1$
(Fig. 3d, left four lanes) and by 22C11 (right four

10 lanes). $\alpha GO1$ (Morishita et al., Eur. J. Biochem.
174:87-94, 1988) was provided by Dr. Tomiko Asano;
similar results were obtained when antibody GC/2 was
substituted. The control lanes are 22C11-precipitate
exposed to G_o in the absence of Sf9 membranes.

15 Approximately 1/10-1/20 (0.05-0.1 μg/tube) of the
reconstituted G_o was precipitated, together with a
comparable amount ($\approx$0.1 μg/tube) of APP. Easily
detectable amounts of $G_o\alpha$ were present in the final
precipitate when G_o was mixed with 22C11-precipitates

20 from Sf9-ΔC or Sf9-APP695 membranes, but essentially no
$G_o\alpha$ was found in the final precipitate from Sf9-ΔN
membranes. Thus, formation of an APP-G_o complex requires
the peptide 20 region, residues 657-676 (SEQ ID NO: 1).

        In the experiment illustrated in Fig. 3e, 22C11-

25 precipitates from Sf9-APP_{695} membranes (100 μg protein
each) were incubated with activated G_o (lanes 2 and 4) or
unactivated G_o (lanes 1 and 3); the final precipitates
(left panel) and supernatants (right panel) were analyzed
by simultaneous immunoblotting with 22C11 and $\alpha GO1$

30 antibodies. Activation of G_o was carried out by
incubating G_o in 20 mM Hepes/NaOH (pH 7.4), 1 mM EDTA, 2
mM $MgCl_2$, and 1 μM GTPγS overnight at room temperature.
When G_o was incubated with GTPγS, no $G_o\alpha$ associated with
the APP-22C11 complex (Fig. 3e), suggesting that the

- 21 -

activation state of the G protein regulates APP-G_O
association.

   This study suggests that APP functions as a
receptor coupled to G_O through the G_O-activator
5 cytoplasmic domain His[657]-Lys[676] (SEQ ID NO: 1). APP has
a point mutation in at least one form of familial
Alzheimer's disease (Goate et al., Nature 349:704-706,
1991). A structural alteration of APP is therefore
thought to be one cause of Alzheimer's disease, although
10 it remains unknown how the mutation might produce the
disease. One novel possibility suggested by this study
is that the cytoplasmic, C-terminal fragment of APP is
pathogenic. It has been suggested (Abraham et al.,
Biotechnology 7:147-153, 1989; Shivers et al., EMBO J.
15 7:1365-1370, 1988; Kametani et al., Biomedical Research
10:179-183, 1989) that the residual C-terminal portion of
APP may remain in the cell membrane after abnormal
cleavage of APP to produce β/A4 protein in Alzheimer's
disease neurons. By analogy with the oncogenic
20 transformation of c-erb B into v-erb B, such a structural
alteration of APP may alter its function and prompt APP
to constitutively activate G_O. This hypothesis is
consistent with the study (Yanker et al., Science
245:417-420, 1989) indicating that recombinant expression
25 of the C-terminal 105-residue portion of APP in neuronal
cells evokes cell death, and with the reports that G_O
activity is linked to neuronal growth cone motility
(Strittmatter et al., BioEssays 13:127-134, 1990), axon
and dendrite formation (Granneman et al., J.
30 Neurochemistry 54:1995-2001, 1990), and memory (Guillen
et al., EMBO J. 9:1449-1455, 1990). This study suggests
that Alzheimer's disease is a disorder of an APP-G_O
signalling system caused by structural alterations of
APP.

- 22 -

Example 1

The screening method of the invention can be carried out as follows:

The assay used can be a very simple cell-free
5 assay employing a first polypeptide consisting essentially of the couplone, or G_o-binding portion, of APP (SEQ ID NO: 1) and a second polypeptide consisting essentially of an APP-binding portion of G_o. This APP-binding portion of G_o may be the 15-residue segment
10 identified as the anticouplone portion of G_o (SEQ ID NO: 3), or it may be one or both of the two flanking regions, residues 1-3 (SEQ ID NO: 4) and residues 19-36 (SEQ ID NO: 5) of G_o. Alternatively, longer portions, or all, of APP and/or G_o can be used, or the appropriate
15 portions of APP and/or G_o can be linked to other polypeptides to form hybrid polypeptides with characteristics (such as altered immunoreactivity or enzymatic activity) that would improve detection of the endpoint of the assay. The assay is carried out by
20 contacting the APP-based polypeptide with the G_o-based polypeptide in the presence of a candidate compound, in parallel with a control assay containing no candidate compound, and determining whether the candidate compound inhibits co-immunoprecipitation of the first and second
25 polypeptides (using either an antibody specific for the first polypeptide or an antibody specific for the second polypeptide). Alternatively, activation of the second (G_o) polypeptide may be the measured criterion: if so, the second polypeptide must include the GTP-binding
30 region of G_o (SEQ ID NO: 10), and GTP or an appropriate non-hydrolyzable analog thereof (such as GTPγS or Gpp(NH)p) must be included in the assay. The assay may also be carried out using phospholipid vesicles prepared by standard methods (e.g., as described by Nishimoto et
35 al., J. Biol. Chem. 264:14029-14038, 1989), provided that

- 23 -

the first (APP) polypeptide includes a region of
hydrophobic amino acids [such as all (SEQ ID NO: 8) or a
portion (e.g., SEQ ID NO: 7) of the transmembrane region
of APP) that permit it to be anchored in the phospholipid
5   bilayer. Alternatively, the assay may be carried out
using intact cells or red cell ghosts which contain APP
and G_o, or appropriate portions thereof. The cells may
express the first and second polypeptides naturally or by
virtue of genetic engineering, or the polypeptides may be
10   introduced directly into the cells or ghosts by standard
means.

Example 2
        The progress of Alzheimer's disease may be halted
or reversed by treating a patient with a compound which
15   diminishes the activation of neural G_o by truncated APP.
Such a compound may be identified in a screening assay as
described above, or may consist essentially of a
polypeptide containing the amino acid sequence of (a) the
couplone region of APP (SEQ ID NO: 1), (b) the
20   anticouplone region of G_o (SEQ ID NO: 3), or (c) the APP-
associating region(s) of G_o (SEQ ID NO: 4 and/or 5), or a
combination of (b) and (c). Such polypeptides may be
produced in quantity by standard recombinant means, or by
standard synthetic techniques. To minimize proteolytic
25   degradation *in vivo*, the carboxy and amino termini may be
derivatized (e.g., with ester or amide groups), some or
all of the amino acids may be replaced with D-amino
acids, or particularly sensitive peptide linkages may be
substituted with non-peptide bonds using standard
30   methodology. To improve penetration of the blood-brain
barrier (BBB), the polypeptides may be altered to
increase lipophilicity (e.g., by esterification to a
bulky lipophilic moiety such as cholesteryl) or to supply
a cleavable "targetor" moiety that enhances retention on

- 24 -

the brain side of the barrier (Bodor et al., Science
257:1698-1700, 1992). Alternatively, the polypeptide may
be linked to an antibody to the transferrin receptor, in
order to exploit that receptor's role in transporting
5 iron across the blood-brain barrier, as taught by Friden
et al., Science 259:373-377, 1993. It is expected that
an intravenous dosage equivalent to approximately 1 to
100 μmoles of the polypeptide of the invention per kg per
day, or an intrathecally administered dosage of
10 approximately 0.1 to 50 μmoles per kg per day, will be
effective in blocking activation of G_o in an Alzheimer's
patient. If the polypeptide is sufficiently protected
from proteolytic degradation, as described above, it may
also be administered orally in appropriately higher
15 doses. Alternatively, the compound may be incorporated
into a slow-release implant to ensure a relatively
constant supply of the therapeutic to the patient's
brain.

- 25 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Nishimoto, Ikuo

    (ii) TITLE OF INVENTION: ALZHEIMER'S DISEASE THERAPEUTICS

    (iii) NUMBER OF SEQUENCES: 30

    (iv) CORRESPONDENCE ADDRESS:

        (A) ADDRESSEE: Fish & Richardson
        (B) STREET: 225 Franklin Street
        (C) CITY: Boston
        (D) STATE: Massachusetts
        (E) COUNTRY: U.S.A.
        (F) ZIP: 02110-2804

    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
        (B) COMPUTER: IBM PS/2 Model 50Z or 55SX
        (C) OPERATING SYSTEM: MS-DOS (Version 5.0)
        (D) SOFTWARE: WordPerfect (Version 5.1)

    (vi) CURRENT APPLICATION DATA:

        (A) APPLICATION NUMBER: 08/019,208
        (B) FILING DATE: February 18, 1993
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:

        (A) APPLICATION NUMBER:
        (B) FILING DATE:

    (viii) ATTORNEY/AGENT INFORMATION:

        (A) NAME: Clark, Paul T.
        (B) REGISTRATION NUMBER: 30,162
        (C) REFERENCE/DOCKET NUMBER: 00786/154001

    (ix) TELECOMMUNICATION INFORMATION:

        (A) TELEPHONE: (617) 542-5070
        (B) TELEFAX: (617) 542-8906
        (C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

- 26 -

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
 1   5   10   15

His Leu Ser Lys
          20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH: 1910
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: double
                (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGTGGCAGGG AAGGGGCCAC C ATG GGA TGT ACG CTG AGC GCA GAG GAG AGA   51
                        Met Gly Cys Thr Leu Ser Ala Glu Glu Arg
                         1   5   10

GCC GCC CTC GAG CGG AGC AAG GCG ATT GAG AAA AAC CTA AAA GAA GAT   99
Ala Ala Leu Glu Arg Ser Lys Ala Ile Glu Lys Asn Leu Lys Glu Asp
                15   20   25

GGC ATC AGC GCC GCC AAA GAC GTG AAA TTA CTC CTG CTG GGG GCT GGA   147
Gly Ile Ser Ala Ala Lys Asp Val Lys Leu Leu Leu Leu Gly Ala Gly
             30   35   40

GAA TCA GGA AAA AGC ACC ATT GTG AAG CAG ATG AAG ATC ATC CAT GAA   195
Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Lys Ile Ile His Glu
         45   50   55

GAT GGC TTC TCT GGG GAA GAC GTG AAG CAG TAC AAG CCT GTG GTC TAC   243
Asp Gly Phe Ser Gly Glu Asp Val Lys Gln Tyr Lys Pro Val Val Tyr
     60   65   70

AGC AAC ACC ATC CAG TCT CTG GCG GCC ATT GTC CGG GCC ATG GAC ACT   291
Ser Asn Thr Ile Gln Ser Leu Ala Ala Ile Val Arg Ala Met Asp Thr
 75   80   85   90

TTG GGC GTG GAG TAT GGT GAC AAG GAG AGG AAG ACG GAC TCC AAG ATG   339
Leu Gly Val Glu Tyr Gly Asp Lys Glu Arg Lys Thr Asp Ser Lys Met
                 95   100   105

GTG TGT GAC GTG GTG AGT CGT ATG GAA GAC ACT GAA CCG TTC TCT GCA   387
Val Cys Asp Val Val Ser Arg Met Glu Asp Thr Glu Pro Phe Ser Ala
             110   115   120

GAA CTT CTT TCT GCC ATG ATG CGA CTC TGG GGC GAC TCG GGG ATC CAG   435
Glu Leu Leu Ser Ala Met Met Arg Leu Trp Gly Asp Ser Gly Ile Gln
         125   130   135

GAG TGC TTC AAC CGA TCT CGG GAG TAT CAG CTC AAT GAC TCT GCC AAA   483
Glu Cys Phe Asn Arg Ser Arg Glu Tyr Gln Leu Asn Asp Ser Ala Lys
     140   145   150

TAC TAC CTG GAC AGC CTG GAT CGG ATT GGA GCC GGT GAC TAC CAG CCC   531
Tyr Tyr Leu Asp Ser Leu Asp Arg Ile Gly Ala Gly Asp Tyr Gln Pro
155   160   165   170

- 27 -

```
ACT GAG CAG GAC ATC CTC CGA ACC AGA GTC AAA ACA ACT GGC ATC GTA        579
Thr Glu Gln Asp Ile Leu Arg Thr Arg Val Lys Thr Thr Gly Ile Val
            175                 180                 185

GAA ACC CAC TTC ACC TTC AAG AAC CTC CAC TTC AGG CTG TTT GAC GTC        627
Glu Thr His Phe Thr Phe Lys Asn Leu His Phe Arg Leu Phe Asp Val
            190                 195                 200

GGG GGC CAG CGA TCT GAA CGC AAG AAG TGG ATC CAC TGC TTT GAG GAT        675
Gly Gly Gln Arg Ser Glu Arg Lys Lys Trp Ile His Cys Phe Glu Asp
            205                 210                 215

GTC ACG GCC ATC ATC TTC TGT GTC GCA CTC AGC GGC TAT GAC CAG GTG        723
Val Thr Ala Ile Ile Phe Cys Val Ala Leu Ser Gly Tyr Asp Gln Val
            220                 225                 230

CTC CAC GAG GAC GAA ACC ACG AAC CGC ATG CAC GAG TCT CTC ATG CTC        771
Leu His Glu Asp Glu Thr Thr Asn Arg Met His Glu Ser Leu Met Leu
235                 240                 245                 250

TTC GAC TCC ATC TGT AAC AAC AAG TTT TTC ATT GAT ACC TCC ATC ATC        819
Phe Asp Ser Ile Cys Asn Asn Lys Phe Phe Ile Asp Thr Ser Ile Ile
                255                 260                 265

CTC TTC CTC AAC AAG AAA GAC CTC TTT GGC GAG AAG ATT AAG AAG TCA        867
Leu Phe Leu Asn Lys Lys Asp Leu Phe Gly Glu Lys Ile Lys Lys Ser
            270                 275                 280

CCC TTG ACC ATC TGC TTT CCC GAA TAC CCA GGC TCC AAC ACC TAT GAA        915
Pro Leu Thr Ile Cys Phe Pro Glu Tyr Pro Gly Ser Asn Thr Tyr Glu
            285                 290                 295

GAT GCA GCT GCC TAC ATC CAA ACA CAG TTT GAA AGC AAA AAC CGC TCA        963
Asp Ala Ala Ala Tyr Ile Gln Thr Gln Phe Glu Ser Lys Asn Arg Ser
            300                 305                 310

CCC AAC AAA GAA ATT TAC TGT CAC ATG ACT TGT GCC ACA GAC ACG AAT       1011
Pro Asn Lys Glu Ile Tyr Cys His Met Thr Cys Ala Thr Asp Thr Asn
315                 320                 325                 330

AAT ATC CAG GTG GTA TTC GAC GCC GTC ACC GAC ATC ATC ATT GCC AAC       1059
Asn Ile Gln Val Val Phe Asp Ala Val Thr Asp Ile Ile Ile Ala Asn
                335                 340                 345

AAT CTC CGG GGC TGC GGC TTG TAC TGACCTCTTG TCCTGTATAG CAACCTATTT      1113
Asn Leu Arg Gly Cys Gly Leu Tyr
            350

GACTGCTTCA TGGACTCTTT GCTGTTGATG TTGATCTCCT GGTAGCATGA CCTTTGGCCT     1173

TTGTAAGACA CACAGCCTTT CTGTACCAAG CCCCTGTCTA ACCTACGACC CCAGAGTGAC     1233

TGACGGCTGT GTATTTCTGT AGAATGCTGT AGAATACAGT TTTAGTTGAG TCTTTACATT     1293

TAGAACTTGA AAGGATTTTA AAAAACAAAA CAAAAACCAT TTCTCATGTG CTTTGTAGCT     1353

TTAAAAGAAA AAAGGAAAAC TCACCATTTA ATCCATATTT CCTTTTTATT TTGAAGTTTA     1413

AAAAAAAAAT GTCTGTACCC ACACCCTCCC CCTTCCCCAC CTCAGCAGAA CTGGGGCTGG     1473

CACACAGAGG CAGTGCTGGG CCTGGCGCCT CCCAGGGCTT CTGTGCAGCC CATGGCTGGT     1533

GGGAACATGT CAGGCTAGTC TGTCTAGAAG GCCACTGGCC ACTGTACCCA CCCTTCCCCA     1593
```

- 28 -

TGCCTGTGGG CTGCCCAGAC ACCTCATATA CCACCAGGCA GTGGCAGCTC CGCCCTGCTC   1653

AGCCATGCGA CTCCAAACAC ACTCAAAGTT TGCGTAGAAA AAGCACAGCT CTGGCAGGGG   1713

TAGCTGCCAC AGACAACGCT CATCACCTAT AGAAATCCAG CCCTATAGAA GCAATTCACC   1773

CAGCCCCTTC CTACACTCCC TTTGTGTTGT TAACTTTTTG GTTTTTCTGG TCCTAGTGAG   1833

TGCCTCCCAT GCATACCTGA CCAGCTCTGC CAGTGTCTGG GGTCTGGGGA ACAGGGGTTG   1893

TGTGGTTTGG TTTTTGG   1910

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 15
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asp Ala Val Thr Asp Ile Ile Ile Ala Lys Asn Leu Arg Gly Cys
1   5   10   15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 3
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Gly Cys
1

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ile Glu Lys Asn Leu Lys Glu Asp Gly Ile Ser Ala Ala Lys Asp Val
1   5   10   15

Lys Leu

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

- 29 -

(i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 47
      (B) TYPE: amino acid
      (C) STRANDEDNESS:
      (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp
 1               5                  10                      15

Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn
                20                  25                  30

Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
        35                  40                  45
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 10
      (B) TYPE: amino acid
      (C) STRANDEDNESS:
      (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
Thr Val Ile Val Ile Thr Leu Val Met Leu
 1               5                  10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 24
      (B) TYPE: amino acid
      (C) STRANDEDNESS:
      (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
 1               5                  10                      15

Ile Val Ile Thr Leu Val Met Leu
                20
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 2085
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: double
      (D) TOPOLOGY: linear

- 30 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
ATG CTG CCC GGT TTG GCA CTG CTC CTG CTG GCC GCC TGG ACG GCT CGG        48
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
1               5                   10                  15

GCG CTG GAG GTA CCC ACT GAT GGT AAT GCT GGC CTG CTG GCT GAA CCC        96
Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
                20                  25                  30

CAG ATT GCC ATG TTC TGT GGC AGA CTG AAC ATG CAC ATG AAT GTC CAG       144
Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
                35                  40                  45

AAT GGG AAG TGG GAT TCA GAT CCA TCA GGG ACC AAA ACC TGC ATT GAT       192
Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
        50                  55                  60

ACC AAG GAA GGC ATC CTG CAG TAT TGC CAA GAA GTC TAC CCT GGA CTG       240
Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Gly Leu
65                  70                  75                  80

CAG ATC ACC AAT GTG GTA GAA GCC AAC CAA CCA GTG ACC ATC CAG AAC       288
Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                85                  90                  95

TGG TGC AAG CGG GGC CGC AAG CAG TGC AAG ACC CAT CCC CAC TTT GTG       336
Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
                100                 105                 110

ATT CCC TAC CGC TGC TTA GTT GGT GAG TTT GTA AGT GAT GCC CTT CTC       384
Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
                115                 120                 125

GTT CCT GAC AAG TGC AAA TTC TTA CAC CAG GAG AGG ATG GAT GTT TGC       432
Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
        130                 135                 140

GAA ACT CAT CTT CAC TGG CAC ACC GTC GCC AAA GAG ACA TGC AGT GAG       480
Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

AAG AGT ACC AAC TTG CAT GAC TAC GGC ATG TTG CTG CCC TGC GGA ATT       528
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165                 170                 175

GAC AAG TTC CGA GGG GTA GAG TTT GTG TGT TGC CCA CTG GCT GAA GAA       576
Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
                180                 185                 190

AGT GAC AAT GTG GAT TCT GCT GAT GCG GAG GAG GAT GAC TGC GAT GTC       624
Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Cys Asp Val
                195                 200                 205

TGG TGG GGC GGA GCA GAC ACA GAC TAT GCA GAT GGG AGT GAA GAC AAA       672
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
        210                 215                 220

GTA GTA GAA GTA GCA GAG GAG GAA GAA GTG GCT GAG GTG GAA GAA GAA       720
Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                 230                 235                 240
```

- 31 -

```
GAA GCC GAT GAT GAC GAG GAC GAT GAG GAT GGT GAT GAG GTA GAG GAA        768
Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
            245                 250                 255

GAG GCT GAG GAA CCC TAC GAA GAA GCC ACA GAG AGA ACC ACC AGC ATT        816
Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270

GCC ACC ACC ACC ACC ACC ACC ACA GAG TCT GTG GAA GAG GTG GTT CGA        864
Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
            275                 280                 285

GTT CCT ACA ACA GCA GCC AGT ACC CCT GAT GCC GTT GAC AAG TAT CTC        912
Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
            290                 295                 300

GAG ACA CCT GGG GAT GAG AAT GAA CAT GCC CAT TTC CAG AAA GCC AAA        960
Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305                 310                 315                 320

GAG AGG CTT GAG GCC AAG CAC CGA GAG AGA ATG TCC CAG GTC ATG AGA       1008
Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
            325                 330                 335

GAA TGG GAA GAG GCA GAA CGT CAA GCA AAG AAC TTG CCT AAA GCT GAT       1056
Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
            340                 345                 350

AAG AAG GCA GTT ATC CAG CAT TTC CAG GAG AAA GTG GAA TCT TTG GAA       1104
Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
            355                 360                 365

CAG GAA GCA GCC AAC GAG AGA CAG CAG CTG GTG GAG ACA CAC ATG GCC       1152
Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
            370                 375                 380

AGA GTG GAA GCC ATG CTC AAT GAC CGC CGC CGC CTG GCC CTG GAG AAC       1200
Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385                 390                 395                 400

TAC ATC ACC GCT CTG CAG GCT GTT CCT CCT CGG CCT CGT CAC GTG TTC       1248
Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
            405                 410                 415

AAT ATG CTA AAG AAG TAT GTC CGC GCA GAA CAG AAG GAC AGA CAG CAC       1296
Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420                 425                 430

ACC CTG AAG CAT TTC GAG CAT GTG CGC ATG GTG GAT CCC AAG AAA GCC       1344
Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
            435                 440                 445

GCT CAG ATC CGG TCC CAG GTT ATG ACA CAC CTC CGT GTG ATT TAT GAG       1392
Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
            450                 455                 460

CGC ATG AAT CAG TCT CTC TCC CTG CTC TAC AAC GTG CCT GCA GTG GCC       1440
Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
465                 470                 475                 480

GAG GAG ATT CAG GAT GAA GTT GAT GAG CTG CTT CAG AAA GAG CAA AAC       1488
Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
            485                 490                 495
```

- 32 -

```
TAT TCA GAT GAC GTC TTG GCC AAC ATG ATT AGT GAA CCA AGG ATC AGT        1536 .
Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
            500                 505                 510

TAC GGA AAC GAT GCT CTC ATG CCA TCT TTG ACC GAA ACG AAA ACC ACC        1584
Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
            515                 520                 525

GTG GAG CTC CTT CCC GTG AAT GGA GAG TTC AGC CTG GAC GAT CTC CAG        1632
Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
            530                 535                 540

CCG TGG CAT TCT TTT GGG GCT GAC TCT GTG CCA GCC AAC ACA GAA AAC        1680
Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
545                 550                 555                 560

GAA GTT GAG CCT GTT GAT GCC CGC CCT GCT GCC GAC CGA GGA CTG ACC        1728
Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
            565                 570                 575

ACT CGA CCA GGT TCT GGG TTG ACA AAT ATC AAG ACG GAG GAG ATC TCT        1776
Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
            580                 585                 590

GAA GTG AAG ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT        1824
Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
            595                 600                 605

CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA        1872
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
            610                 615                 620

GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA GTG        1920
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625                 630                 635                 640

ATC GTC ATC ACC TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC ATT        1968
Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
            645                 650                 655

CAT CAT GGT GTG GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG CGC        2016
His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
            660                 665                 670

CAC CTG TCC AAG ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC AAG        2064
His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
            675                 680                 685

TTC TTT GAG CAG ATG CAG AAC                                            2085
Phe Phe Glu Gln Met Gln Asn
690                 695
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 16
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

- 33 -

```
Lys Leu Leu Leu Leu Gly Ala Gly Glu Ser Gly Lys Ser Thr Ile Val
 1               5                 10                    15
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 10
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu
 1               5                 10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 10
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr
 1               5                 10
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 19
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln
 1               5                 10                    15
Met Gln Asn
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 19
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear
```

- 34 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His
 1               5                   10                  15

Leu Ser Lys

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:      15:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH:              18
                (B) TYPE:                amino acid
                (C) STRANDEDNESS:
                (D) TOPOLOGY:            linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu
 1               5                   10                  15

Ser Lys

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:      16:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH:              15
                (B) TYPE:                amino acid
                (C) STRANDEDNESS:
                (D) TOPOLOGY:            linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu
 1               5                   10                  15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:      17:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH:              26
                (B) TYPE:                amino acid
                (C) STRANDEDNESS:
                (D) TOPOLOGY:            linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala Ala
 1               5                   10                  15

Val Thr Pro Glu Glu Arg His Leu Ser Lys
              20                  25

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:      18:

- 35 -

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH:                  30
                (B) TYPE:                    amino acid
                (C) STRANDEDNESS:
                (D) TOPOLOGY:                linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:


Thr Val Ile Val Ile Thr Leu Val Met Leu His His Gly Val Val Glu
 1               5                   10                  15

Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys
            20                  25                  30

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     19:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH:                  36
                (B) TYPE:                    nucleic acid
                (C) STRANDEDNESS:            single
                (D) TOPOLOGY:                linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:


CCACGCAGGA TCACGGGATC CATGCTGCCC AGCTTG                 36


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     20:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH:                  6
                (B) TYPE:                    nucleic acid
                (C) STRANDEDNESS:            single
                (D) TOPOLOGY:                linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:


GGATCC                                                  6


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     21:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH:                  36
                (B) TYPE:                    nucleic acid
                (C) STRANDEDNESS:            single
                (D) TOPOLOGY:                linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:


CAGTACACAT CCATCTGATG ACATCATGGC GTGGTG                 36

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     22:

- 36 -

(i) SEQUENCE CHARACTERISTICS:

   (A) LENGTH:      35
   (B) TYPE:       nucleic acid
   (C) STRANDEDNESS:   single
   (D) TOPOLOGY:    linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:


CGCCATCTCT CCAGTGATGA ATGCAGCAGA ACGGA      35


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:  23:

  (i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH:      656
    (B) TYPE:       amino acid
    (C) STRANDEDNESS:
    (D) TOPOLOGY:    linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:


Met Leu Pro Gly Leu Ala Leu Leu Leu Ala Ala Trp Thr Ala Arg
1     5      10      15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
   20      25      30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
   35      40      45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
  50      55      60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Gly Leu
65      70      75      80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
     85      90      95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
   100     105     110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
   115     120     125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
  130     135     140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145      150     155     160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
     165     170     175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
   180     185     190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Cys Asp Val
   195     200     205

- 37 -

```
Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210             215             220

Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225             230             235                 240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
            245             250             255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
        260             265             270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
    275             280             285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
    290             295             300

Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305             310             315             320

Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
            325             330             335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
        340             345             350

Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
        355             360             365

Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
    370             375             380

Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385             390             395             400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
            405             410             415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420             425             430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
    435             440             445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
    450             455             460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
465             470             475             480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
            485             490             495

Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
            500             505             510

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
        515             520             525

Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
530             535             540
```

- 38 -

Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
545   550   555   560

Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
                565   570   575

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
                580   585   590

Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
        595   600   605

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
    610   615   620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625   630   635   640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
                645   650   655

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 24:

        (i) SEQUENCE CHARACTERISTICS:

            (A) LENGTH: 676
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Met Leu Pro Gly Leu Ala Leu Leu Leu Ala Ala Trp Thr Ala Arg
1   5   10   15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
                20   25   30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
        35   40   45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
    50   55   60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Gly Leu
65   70   75   80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                85   90   95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100   105   110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
            115   120   125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
        130   135   140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145   150   155   160

```
Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
            165             170             175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180             185             190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Cys Asp Val
            195             200             205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210             215             220

Val Val Glu Val Ala Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225             230             235             240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
            245             250             255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260             265             270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
    275             280             285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
    290             295             300

Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305             310             315             320

Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
            325             330             335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
            340             345             350

Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
    355             360             365

Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
    370             375             380

Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385             390     .       395             400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
            405             410             415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420             425             430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
            435             440             445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
    450             455             460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
465             470             475             480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
            485             490             495
```

- 40 -

```
Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
        500             505             510

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
        515             520             525

Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
    530             535             540

Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
545             550             555             560

Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
        565             570             575

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
        580             585             590

Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
        595             600             605

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
    610             615             620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625             630             635             640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
        645             650             655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
        660             665             670

His Leu Ser Lys
        675
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 25:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 58
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Ala Asp Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val Asp
  1             5               10              15

Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser Gly
        20              25              30

Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp Ala
        35              40              45

Glu Phe Arg His Asp Ser Gly Tyr Glu Val
    50              55
```

- 41 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 26:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 56
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Val Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser
 1   5   10   15

Ile His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu
            20   25   30

Arg His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr
            35   40   45

Lys Phe Phe Glu Gln Met Gln Asn
    50   55

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 27:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 695
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
 1   5   10   15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
            20   25   30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
            35   40   45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
    50   55   60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Gly Leu
65   70   75   80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                85   90   95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100   105   110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
            115   120   125

- 42 -

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130   135   140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145   150   155   160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
            165   170   175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180   185   190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Cys Asp Val
        195   200   205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210   215   220

Val Val Glu Val Ala Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225   230   235   240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
            245   250   255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
        260   265   270

Ala Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
    275   280   285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
    290   295   300

Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305   310   315   320

Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
        325   330   335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
        340   345   350

Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
        355   360   365

Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
    370   375   380

Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385   390   395   400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
            405   410   415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420   425   430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
        435   440   445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
    450   455   460

- 43 -

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
465   470   475   480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
              485   490   495

Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
              500   505   510

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
              515   520   525

Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
              530   535   540

Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
545   550   555   560

Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
              565   570   575

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
              580   585   590

Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
        595   600   605

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
        610   615   620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625   630   635   640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
              645   650   655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
              660   665   670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
        675   680   685

Phe Phe Glu Gln Met Gln Asn
690   695

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 28:

     (i) SEQUENCE CHARACTERISTICS:

         (A) LENGTH: 2274
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: double
         (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCTGTGGCAG GGAAGGGGCC ACC ATG GGA TGT ACG CTG AGC GCA GAG GAG   50
                          Met Gly Cys Thr Leu Ser Ala Glu Glu
                           1   5

AGA GCC GCC CTC GAG CGG AGC AAG GCG ATT GAG AAA AAC CTC AAA GAA   98
Arg Ala Ala Leu Glu Arg Ser Lys Ala Ile Glu Lys Asn Leu Lys Glu
 10   15   20   25

- 44 -

```
GAT GGC ATC AGC GCC GCC AAA GAC GTG AAA TTA CTC CTG CTG GGG GCT    146
Asp Gly Ile Ser Ala Ala Lys Asp Val Lys Leu Leu Leu Leu Gly Ala
            30              35                      40

GGA GAA TCA GGA AAA AGC ACC ATT GTG AAG CAG ATG AAG ATC ATC CAT    194
Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Lys Ile Ile His
            45              50                      55

GAA GAT GGC TTC TCT GGG GAA GAC GTG AAG CAG TAC AAG CCT GTG GTC    242
Glu Asp Gly Phe Ser Gly Glu Asp Val Lys Gln Tyr Lys Pro Val Val
            60              65              70

TAC AGC AAC ACC ATC CAG TCT CTG GCG GCC ATT GTC CGG GCC ATG GAC    290
Tyr Ser Asn Thr Ile Gln Ser Leu Ala Ala Ile Val Arg Ala Met Asp
    75              80                      85

ACT TTG GGC GTG GAG TAT GGT GAC AAG GAG AGG AAG ACG GAC TCC AAG    338
Thr Leu Gly Val Glu Tyr Gly Asp Lys Glu Arg Lys Thr Asp Ser Lys
90              95              100             105

ATG GTG TGT GAC GTG GTG AGT CGT ATG GAA GAC ACT GAA CCG TTC TCT    386
Met Val Cys Asp Val Val Ser Arg Met Glu Asp Thr Glu Pro Phe Ser
            110             115             120

GCA GAA CTT CTT TCT GCC ATG ATG CGA CTC TGG GGC GAC TCG GGG ATC    434
Ala Glu Leu Leu Ser Ala Met Met Arg Leu Trp Gly Asp Ser Gly Ile
            125             130             135

CAG GAG TGC TTC AAC CGA TCT CGG GAG TAT CAG CTC AAT GAC TCT GCC    482
Gln Glu Cys Phe Asn Arg Ser Arg Glu Tyr Gln Leu Asn Asp Ser Ala
            140             145             150

AAA TAC TAC CTG GAC AGC CTG GAT CGG ATT GGA GCC GGT GAC TAC CAG    530
Lys Tyr Tyr Leu Asp Ser Leu Asp Arg Ile Gly Ala Gly Asp Tyr Gln
    155             160             165

CCC ACT GAG CAG GAC ATC CTC CGA ACC AGA GTC AAA ACA ACT GGC ATC    578
Pro Thr Glu Gln Asp Ile Leu Arg Thr Arg Val Lys Thr Thr Gly Ile
170             175             180             185

GTA GAA ACC CAC TTC ACC TTC AAG AAC CTC CAC TTC AGG CTG TTT GAC    626
Val Glu Thr His Phe Thr Phe Lys Asn Leu His Phe Arg Leu Phe Asp
            190             195             200

GTC GGG GGC CAG CGA TCT GAA CGC AAG AAG TGG ATC CAC TGC TTT GAG    674
Val Gly Gly Gln Arg Ser Glu Arg Lys Lys Trp Ile His Cys Phe Glu
            205             210             215

GAT GTC ACG GCC ATC ATC TTC TGT GTC GCA CTC AGC GGC TAT GAC CAG    722
Asp Val Thr Ala Ile Ile Phe Cys Val Ala Leu Ser Gly Tyr Asp Gln
            220             225             230

GTG CTC CAC GAG GAC GAA ACC ACG AAC CGC ATG CAC GAA TCC CTG AAG    770
Val Leu His Glu Asp Glu Thr Thr Asn Arg Met His Glu Ser Leu Lys
    235             240             245

CTC TTC GAC AGC ATC TGC AAC AAC AAG TGG TTC ACA GAC ACA TCT ATT    818
Leu Phe Asp Ser Ile Cys Asn Asn Lys Trp Phe Thr Asp Thr Ser Ile
250             255             260             265

ATC CTG TTT CTC AAC AAG AAG GAC ATA TTT GAG GAG AAG ATC AAG AAG    866
Ile Leu Phe Leu Asn Lys Lys Asp Ile Phe Glu Glu Lys Ile Lys Lys
            270             275             280
```

- 45 -

```
TCC CCA CTC ACC ATC TGC TTT CCT GAA TAC ACA CGC CCC AGT GCC TTC          914
Ser Pro Leu Thr Ile Cys Phe Pro Glu Tyr Thr Gly Pro Ser Ala Phe
            285             290             295

ACA GAA GCT GTG GCT CAC ATC CAA GGG CAG TAT GAG AGT AAG AAT AAG          962
Thr Glu Ala Val Ala His Ile Gln Gly Gln Tyr Glu Ser Lys Asn Lys
            300             305             310

TCA GCT CAC AAG GAA GTC TAC AGC CAT GTC ACC TGT GCC ACG GAC ACC         1010
Ser Ala His Lys Glu Val Tyr Ser His Val Thr Cys Ala Thr Asp Thr
            315             320             325

AAC AAC ATC CAA TTC GTC TTT GAT GCC GTG ACA GAT GTC ATC ATC GCC         1058
Asn Asn Ile Gln Phe Val Phe Asp Ala Val Thr Asp Val Ile Ile Ala
330             335             340             345

AAA AAC CTA CGG GGC TGT GGA CTC TAC TGAGCCCTGG CCTCCTACCC               1105
Lys Asn Leu Arg Gly Cys Gly Leu Tyr
            350
```

```
AGCCTGCCAC TCACTCCTCC CCTGGACCCA GAGCTCTGTC ACTGCTCAGA TGCCCTGTTA       1165

ACTGAAGAAA ACCTGGAGGC TAGCCTTGGG GGCAGGAGGA GGCATCCTTT GAGCATCCCC       1225

ACCCCACCCA ACTTCAGCCT CGTGACACGT GGGAACAGGG TTGGGCAGAG GTGTGGAACA       1285

GCACAAGGCC AGAGACCACG GCATGCCACT TGGGTGCTGC TCACTGGTCA GCTGTGTGTC       1345

TTACACAGAG GCCGAGTGGG CAACACTGCC ATCTGATTCA GAATGGGCAT GCCCTGTCCT       1405

CTGTACCTCT TGTTCAGTGT CCTGGTTTCT CTTCCACCTT GGTGATAGGA TGGCTGGCAG       1465

GAAGGCCCCA TGGAAGGTGC TGCTTGATTA GGGGATAGTC GATGGCATCT CTCAGCAGTC       1525

CTCAGGGTCT GTTTGGTAGA GGGTGGTTTC GTCGACAAAA GCCAACATGG AATCAGGCCA       1585

CTTTTGGGGC GCAAAGACTC AGACTTTGGG GACGGGTTCC CTCCTCCTTC ACTTTGGATC       1645

TTGGCCCCTC TCTGGTCATC TTCCCTTGCC CTTGGGCTCC CCAGGATACT CAGCCCTGAC       1705

TCCCATGGGG TTGGGAATAT CCTTAAGAC TGGCTGACTG CAAAGGTCAC CGATGGAGAA        1765

ACATCCCTGT GCTACAGAAT TGGGGGTGGG ACAGCTGAGG GGGCAGGCGG CTCTTTCCTG       1825

ATAGTTGATG ACAAGCCCTG AGAATGCCAT CTGCTGGCTC CACTCACACG GCTCAACTG        1885

TCCTGGGTGA TAGTGACTTG CCAGGCCACA GGCTGCAGGT CACAGACAGA GCAGGCAAGC       1945

AGCCTTGCAA CTGCAGATTA CTTAGGGAGA AGCATCCTAG CCCCAGCTAA CTTTGGACAG       2005

TCAGCATATG TCCCTGCCAT CCCTAGACAT CTCCAGTCAG CTGGTATCAC AGCCAGTGGT       2065

TCAGACAGGT TTGAATGCTC ATGTGGCAGG GGGCCCGGTA CCCAGCTTTT GTTCCCTTTA       2125

GTGAGGGTTA ATTGCGCGCT TGGGCTAATC ATGGTCATAG CTGTTGGGCG TTGCTGGCGT       2185

TTTTCCATAG GCTCCGCCCC CTGACGAGAT CACAAAAATC GACGCTCAAG TCAGAGGTGG       2245

CGAAACCGAC AGACTATAAG ATACCAGGC                                          2274
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- 46 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 29:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Asp Val Gly Gly Gln Arg Ser Glu Arg Lys Lys Trp Ile His Cys Phe
 1   5   10   15

Glu Asp

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 30:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 12
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Thr Ser Ile Ile Leu Phe Leu Asn Lys Lys Asp Leu
 1   5   10

- 47 -

CLAIMS

1.   A method of identifying a therapeutic useful
for treating or preventing the symptoms of Alzheimer's
disease, which method includes the steps of

5   contacting (a) a first molecule comprising the
couplone portion (SEQ ID NO: 1) of amyloid precursor
protein (APP) with (b) a second molecule comprising an
APP-associating region of G_o (SEQ ID NOs: 3, 4, or 5), in
the presence of a candidate compound; and

10   determining whether said candidate compound
interferes with the association of said first and second
molecules, said interference being an indication that
said candidate compound is a therapeutic useful for
treating Alzheimer's disease.

15   2.   The method of claim 1, wherein said
determining step is accomplished by

         immmunoprecipitating said first molecule with an
antibody specific for APP; and

         detecting the presence or amount of said second
20   molecule which co-precipitates with said first molecule.

         3.   The method of claim 1, wherein said
determining step is accomplished by

         immunoprecipitating said second molecule with an
antibody specific for G_o; and

25   detecting the presence or amount of said first
molecule which co-precipitates with said second molecule.

         4.   The method of claim 1, wherein said first
molecule comprises the portion of APP_{695} from residues 649
to 695 (SEQ ID NO: 6).

- 48 -

5.   The method of claim 1, wherein said first molecule comprises the portion of APP$_{695}$ from residues 639 to 648 (SEQ ID NO: 7).

6.   The method of claim 1, wherein said first molecule comprises the portion of APP$_{695}$ from residues 640 to 695 (SEQ ID NO: 26).

7.   The method of claim 6, wherein said first molecule comprises essentially all of APP$_{695}$ (SEQ ID NO: 27).

8.   The method of claim 1, wherein said second molecule comprises the GTP-binding region of G$_o$ (SEQ ID NO: 10).

9.   The method of claim 8, wherein said second molecule comprises essentially all of G$_o$ (SEQ ID NO: 2).

10.   A method of assaying for a therapeutic useful for treating Alzheimer's disease, which method includes the steps of
        contacting (a) a first molecule comprising the couplone region of APP (SEQ ID NO: 1) with (b) a second molecule comprising an APP-associating region of G$_o$ (SEQ ID NO: 3, 4, or 5), in the presence of a candidate compound; and
        determining whether said candidate compound interferes with the activation of said second molecule by said first molecule, said interference being an indication that said candidate compound is a therapeutic useful for treating Alzheimer's disease.

11.   The method of claim 10, wherein said determining step is accomplished by

- 49 -

contacting said second molecule with a substrate
comprising GTP or an analog of GTP; and
detecting or measuring the binding of said
substrate to said second molecule, wherein said binding
5   is evidence of said activation of said second molecule by
said first molecule.

12.   The method of claim 1, wherein said
contacting step is carried out at a Mg^{2+} concentration
between 1×10^{-7} and 1×10^{-2} M.

10   13.   The method of claim 10, wherein said
contacting step is carried out at a Mg^{2+} concentration
between 1×10^{-7} and 1×10^{-2} M.

14.   The method of claim 1, wherein said
contacting step is carried out in a cell-free system.

15   15.   The method of claim 10, wherein said
contacting step is carried out in a cell-free system.

16.   A system for screening candidate Alzheimer's
disease therapeutics, which system comprises
a first polypeptide comprising a sequence
20   essentially identical to that of peptide 20 (SEQ ID
NO: 1);
a second polypeptide comprising a sequence
essentially identical to the anticouplone sequence of G_o
(SEQ ID NO: 3); and
25   a means for detecting either (a) the association
of said first polypeptide with said second polypeptide,
or (b) the activation of said second polypeptide by said
first polypeptide.

- 50 -

17. A cell-free system for screening candidate Alzheimer's disease therapeutics, which system comprises
     a first polypeptide comprising a sequence essentially identical to that of peptide 20 (SEQ ID

5   NO: 1); and
     a second polypeptide comprising a sequence essentially identical to the anticouplone sequence of G_o (SEQ ID NO: 3).

18. The system of claim 17, wherein said first

10 polypeptide is anchored to a solid material or is in a phospholipid vesicle.

19. The system of claim 17, wherein said second polypeptide further comprises residues 1 to 3 (SEQ ID NO: 4) and 19 to 36 (SEQ ID NO: 5) of G_o.

15   20. The system of claim 19, wherein said second polypeptide comprises G_o1 or G_o2.

21. A method for diminishing the activation of G_o in a neuronal cell by treating the cell with a compound which blocks association of G_o with the cytoplasmic tail

20 of APP.

22. The method of claim 21, wherein the compound is a peptide fragment of G_o or of the cytoplasmic tail of APP.

23. The method of claim 21, wherein said cell is

25 within an animal.

24. The method of claim 23, wherein said animal is a human.

- 51 -

25.   A method for preventing or treating
Alzheimer's disease in a patient, comprising treating the
patient with a compound which blocks association of G_o
with the cytoplasmic tail of APP.

5   26.   A method for preventing or treating
Alzheimer's disease in a patient, comprising treating the
patient with a compound which inhibits activation of
neuronal G_o by the cytoplasmic tail of APP.

27.   A peptide having less than 50 amino acids and
10   comprising the sequence of peptide 20 (SEQ ID NO: 1).

28.   A therapeutic composition comprising the
peptide of claim 27 and a pharmaceutically acceptable
carrier.

29.   A method for identifying a ligand for which
15   APP is a receptor, which method includes the steps of
          providing an APP molecule and a G_o molecule;
          contacting a candidate compound with the
extracellular domain of said APP molecule, the
cytoplasmic tail of said APP molecule being accessible to
20   said G_o molecule, and
          detecting either (a) association of said G_o
molecule with said APP molecule, or (b) activation of
said G_o molecule by said APP molecule, said association
or activation being evidence that said candidate compound
25   is a ligand of APP.

FIG. 1A

FIG. 1B

GTP-γS BINDING
(mol per mol G_o per 2 min.)

FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1F

KDa

97 —

40 —

FIG. 2A   1   2   3   4

40 —

29 —

Mg^{2+} no   100μ   50 m
(M)   1 μ   10m   CONTROL

FIG. 2C

FIG. 2B

FIG. 2D

FIG. 3A

FIG. 3B

695   ATG ... TAA

ΔN   ATG ... TGATGA

ΔC   ATG ... TGATGA

▨ TRANSMEMBRANE   ■ PEPTIDE 20

FIG. 3E

FIG. 3C

97k——

40k——

CONTROL 695   ΔN   ΔC CONTROL 695   ΔN   ΔC

FIG. 3D

```
TGTGGCAGGG AAGGGGCCAC C ATG GGA TGT ACG CTG AGC GCA GAG GAG AGA    51
                         Met Gly Cys Thr Leu Ser Ala Glu Glu Arg
                          1               5                  10

GCC GCC CTC GAG CGG AGC AAG GCG ATT GAG AAA AAC CTA AAA GAA GAT    99
Ala Ala Leu Glu Arg Ser Lys Ala Ile Glu Lys Asn Leu Lys Glu Asp
             15                  20                  25

GGC ATC AGC GCC GCC AAA GAC GTG AAA TTA CTC CTG CTG GGG GCT GGA   147
Gly Ile Ser Ala Ala Lys Asp Val Lys Leu Leu Leu Leu Gly Ala Gly
         30                  35                  40

GAA TCA GGA AAA AGC ACC ATT GTG AAG CAG ATG AAG ATC ATC CAT GAA   195
Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Lys Ile Ile His Glu
     45                  50                  55

GAT GGC TTC TCT GGG GAA GAC GTG AAG CAG TAC AAG CCT GTC GTC TAC   243
Asp Gly Phe Ser Gly Glu Asp Val Lys Gln Tyr Lys Pro Val Val Tyr
 60                  65                  70

AGC AAC ACC ATC CAG TCT CTG GCG GCC ATT GTC CGG GCC ATG GAC ACT   291
Ser Asn Thr Ile Gln Ser Leu Ala Ala Ile Val Arg Ala Met Asp Thr
         75                  80                  85          90

TTG GGC GTG GAG TAT GGT GAC AAG GAG AGG GAC TCC AAG ATG           339
Leu Gly Val Glu Tyr Gly Asp Lys Glu Arg Asp Ser Lys Met
         95                 100                 105

GTG TGT GAC GTG AGT CGT ATG GAA GAC ACT GAA CCG TTC TCT GCA       387
Val Cys Asp Val Ser Arg Met Glu Asp Thr Glu Pro Phe Ser Ala
         110                 115                 120
```

FIG. 4A-1

```
GAA CTT CTT TCT GCC ATG ATG CGA CTC TGG GGC GAC TCG GGG ATC CAG      435
Glu Leu Leu Ser Ala Met Met Arg Leu Trp Gly Asp Ser Gly Ile Gln
        125             130             135

GAG TGC TTC AAC CGA TCT CGG GAG TAT CAG CTC AAT GAC TCT GCC AAA      483
Glu Cys Phe Asn Arg Ser Arg Glu Tyr Gln Leu Asn Asp Ser Ala Lys
        140             145             150

TAC TAC CTG GAC AGC CTG GAT CGG ATT GGA GCC GGT GAC TAC CAG CCC      531
Tyr Tyr Leu Asp Ser Leu Asp Arg Ile Gly Ala Gly Asp Tyr Gln Pro
        155             160             165             170

ACT GAG CAG GAC ATC CTC CGA ACC AGA GTC AAA ACA ACT GGC ATC GTA      579
Thr Glu Gln Asp Ile Leu Arg Thr Arg Val Lys Thr Thr Gly Ile Val
        175             180             185

GAA ACC CAC TTC AAG AAC CTC CAC TTC AGG CTG TTT GAC GTC              627
Glu Thr His Phe Lys Asn Leu His Phe Arg Leu Phe Asp Val
        190             195             200

GGG GGC CAG CGA TCT GAA CGC AAG TGG TGG ATC CAC TGC TTT GAG GAT      675
Gly Gly Gln Arg Ser Glu Arg Lys Trp Lys Ile His Cys Phe Glu Asp
        205             210             215

GTC ACG GCC ATC ATC TTC TGT GTC GCA CTC AGC GGC TAT GAC CAG GTG      723
Val Thr Ala Ile Ile Phe Cys Val Ala Leu Ser Gly Tyr Asp Gln Val
        220             225             230

CTC CAC GAG GAC GAA ACC ACG AAC CGC ATG CAC GAG TCT CTC ATG CTC      771
Leu His Glu Asp Glu Thr Thr Asn Arg Met His Glu Ser Leu Met Leu
        235             240             245             250
```

FIG. 4A-2

```
TTC GAC TCC ATC TGT AAC AAC AAG TTT TTC ATT GAT ACC TCC ATC ATC     819
Phe Asp Ser Ile Cys Asn Asn Lys Phe Phe Ile Asp Thr Ser Ile Ile
                255                     260                     265

CTC TTC AAC AAG AAA GAC CTC TTT GGC GAG AAG ATT AAG AAG TCA         867
Leu Phe Asn Lys Lys Asp Leu Phe Gly Glu Lys Ile Lys Lys Ser
                270                     275                     280

CCC TTG ACC ATC TGC TTT CCC GAA TAC CCA GGC TCC AAC ACC TAT GAA     915
Pro Leu Thr Ile Cys Phe Pro Glu Tyr Pro Gly Ser Asn Thr Tyr Glu
                285                     290                     295

GAT GCA GCT GCC TAC ATC CAA ACA CAG TTT GAA AGC AAA AAC CGC TCA     963
Asp Ala Ala Ala Tyr Ile Gln Thr Gln Phe Glu Ser Lys Asn Arg Ser
                300                     305                     310

CCC AAC AAA GAA ATT TAC TGT CAC ATG ACT TGT GCC ACA GAC ACG AAT     1011
Pro Asn Lys Glu Ile Tyr Cys His Met Thr Cys Ala Thr Asp Thr Asn
                315                     320                     325                     330

AAT ATC CAG GTG GTA TTC GAC GCC GTC ACC GAC ATC ATT GCC AAC         1059
Asn Ile Gln Val Val Phe Asp Ala Val Thr Asp Ile Ile Ala Asn
                335                     340                     345

AAT CTC CGG GGC TTG TAC TGACCTCTTG TCCTGTATAG CAACCTATTT            1113
Asn Leu Arg Gly Leu Tyr
                350
```

FIG. 4A-3

```
GACTGCTTCA TGGACTCTTT GCTGTTGATG TTGATCTCCT GGTAGCATGA CCTTTGGCCT 1173
TTGTAAGACA CACAGCCTTT CTGTACCAAG CCCCTGTCTA ACCTACGACC CCAGAGTGAC 1233
TGACGGCTGT GTATTTCTGT AGAATGCTGT AGAATACAGT TTTAGTTGAG TCTTTACATT 1293
TAGAACTTGA AAGGATTTTA AAAAACAAAA CAAAAACCAT TTCTCATGTG CTTTGTAGCT 1353
TTAAAAGAAA AAAGGAAAAC TCACCATTTA ATCCATATTT CCTTTTTATT TTGAAGTTTA 1413
AAAAAAAAAT GTCTGTACCC ACACCCTCCC CCTTCCCCAC CTCAGCAGAA CTGGGGCTGG 1473
CACACAGAGG CAGTGCTGGG CCTGGCGCCT CCCAGGGCTT CTGTGCAGCC CATGGCTGGT 1533
GGGAACATGT CAGGCTAGTC TGTCTAGAAG GCCACTGGCC ACTGTACCCA CCCTTCCCCA 1593
TGCCTGTGGG CTGCCCAGAC ACCTCATATA CCACCAGGCA GTGGCAGCTC CGCCCTGCTC 1653
AGCCATGCGA CTCCAAACAC ACTCAAAGTT TGCGTAGAAA AAGCACAGCT CTGGCAGGGG 1713
TAGCTGCCAC AGACAACGCT CATCACCTAT AGAAATCCAG CCCTATAGAA GCAATTCACC 1773
CAGCCCCTTC CTACACTCCC TTTGTGTGT  TAACTTTTTG GTTTTTCTGG TCCTAGTGAG 1833
TGCCTCCCAT GCATACCTGA CCAGCTCTGC CAGTGTCTGG GGTCTGGGGA ACAGGGGTTG 1893
TGTGGTTTGG TTTTTGG                                             1910
```

FIG. 4A-4

```
GCTGTGGCAG GGAAGGGGCC ACC ATG GGA TGT ACG CTG AGC GCA GAG GAG      50
                      Met Gly Cys Thr Leu Ser Ala Glu Glu
                       1                               5

AGA GCC GCC CTC GAG CGG AGC GCG ATT GAG AAA AAC CTC AAA GAA        98
Arg Ala Ala Leu Glu Arg Ser Ala Ile Glu Lys Asn Leu Lys Glu
         10                  15                  20          25

GAT GGC ATC AGC GCC AAA GAC GTG AAA TTA CTC CTG GGG GCT          146
Asp Gly Ile Ser Ala Ala Lys Asp Val Lys Leu Leu Leu Gly Ala
             30                  35                  40

GGA GAA TCA GGA AAA AGC ACC ATT GTG AAG CAG ATG AAG ATC ATC CAT  194
Gly Glu Ser Gly Lys Ser Thr Ile Val Lys Gln Met Lys Ile Ile His
         45                  50                  55

GAA GAT GGC TTC TCT GGG GAA GAC GTG AAG TAC AAG CCT GTG GTC      242
Glu Asp Gly Phe Ser Gly Glu Asp Val Lys Gln Tyr Lys Pro Val Val
         60                  65                  70

TAC AGC AAC ACC ATC CAG TCT CTG GCG GCC ATT GTC CGG GCC ATG GAC  290
Tyr Ser Asn Thr Ile Gln Ser Leu Ala Ile Val Arg Ala Met Asp
         75                  80                  85

ACT TTG GGC GTG GAG TAT GGT GAC AAG GAG AGG AAG ACG GAC TCC AAG  338
Thr Leu Gly Val Glu Tyr Gly Asp Lys Glu Arg Lys Thr Asp Ser Lys
         90                  95                  100                 105

ATG GTG TGT GAC GTG AGT CGT ATG GAA GAC ACT GAA CCG TTC TCT      386
Met Val Cys Asp Val Ser Arg Met Glu Asp Thr Glu Pro Phe Ser
         110                 115                 120
```

FIG. 4B-1

```
GCA GAA CTT TCT GCC ATG ATG CGA CTC TGG GGC GAC TCG GGG ATC     434
Ala Glu Leu Ser Ala Met Met Arg Leu Trp Gly Asp Ser Gly Ile
        125             130             135

CAG GAG TGC TTC AAC CGA TCT CGG GAG TAT CAG CTC AAT GAC TCT GCC     482
Gln Glu Cys Phe Asn Arg Ser Arg Glu Tyr Gln Leu Asn Asp Ser Ala
        140             145             150

AAA TAC TAC CTG GAC AGC CTG GAT CGG ATT GGA GCC GGT GAC TAC CAG     530
Lys Tyr Tyr Leu Asp Ser Leu Asp Arg Ile Gly Ala Gly Asp Tyr Gln
        155             160             165

CCC ACT GAG CAG GAC ATC CTC CGA ACC AGA GTC AAA ACA ACT GGC ATC     578
Pro Thr Glu Gln Asp Ile Leu Arg Thr Arg Val Lys Thr Thr Gly Ile
        170             175             180             185

GTA GAA ACC CAC TTC AAG AAC CTC CAC TTC AGG CTG TTT GAC     626
Val Glu Thr His Phe Lys Asn Leu His Phe Arg Leu Phe Asp
        190             195             200

GTC GGG GGC CAG CGA TCT GAA CGC AAG AAG TGG ATC CAC TGC TTT GAG     674
Val Gly Gly Gln Arg Ser Glu Arg Lys Lys Trp Ile His Cys Phe Glu
        205             210             215

GAT GTC ACG GCC ATC TTC TGT GTC GCA CTC AGC GGC TAT GAC CAG     722
Asp Val Thr Ala Ile Phe Cys Val Ala Leu Ser Gly Tyr Asp Gln
        220             225             230

GTG CTC CAC GAG GAA ACC ACG AAC CGC ATG CAC GAA TCC CTG AAG     770
Val Leu His Glu Glu Thr Thr Asn Arg Met His Glu Ser Leu Lys
        235             240             245
```

FIG. 4B-2

```
CTC TTC GAC AGC ATC TGC AAC AAG TGG TTC ACA GAC ACA TCT ATT      818
Leu Phe Asp Ser Ile Cys Asn Asn Lys Trp Phe Thr Asp Thr Ser Ile
250                         255                 260             265

ATC CTG TTT CTC AAC AAG GAC ATA TTT GAG GAG AAG ATC AAG AAG      866
Ile Leu Phe Leu Asn Lys Asp Ile Phe Glu Glu Lys Ile Lys Lys
                270                 275                 280

TCC CCA CTC ACC ATC TGC TTT CCT GAA TAC ACA CGC CCC AGT GCC TTC  914
Ser Pro Leu Thr Ile Cys Phe Pro Glu Tyr Thr Gly Pro Ser Ala Phe
        285                 290                 295

ACA GAA GCT GTG GCT CAC ATC CAA GGG CAG TAT GAG AGT AAG AAT AAG  962
Thr Glu Ala Val Ala His Ile Gln Gly Gln Tyr Glu Ser Lys Asn Lys
        300                 305                 310

TCA GCT CAC AAG GAA GTC TAC AGC CAT GTC ACC TGT GCC ACG GAC ACC  1010
Ser Ala His Lys Glu Val Tyr Ser His Val Thr Cys Ala Thr Asp Thr
        315                 320                 325

AAC AAC ATC CAA TTC GTC TTT GAT GCC GTG ACA GAT GTC ATC ATC GCC  1058
Asn Asn Ile Gln Phe Val Phe Asp Ala Val Thr Asp Val Ile Ile Ala
        330                 335                 340             345

AAA AAC CTA CGG GGC TGT GGA CTC TAC TGAGCCCTGG CCTCCTACCC       1105
Lys Asn Leu Arg Gly Cys Gly Leu Tyr
        350

AGCCTGCCAC TCACTCCTCC CCTGGACCCA GAGCTCTGTC ACTGCTCAGA TGCCCTGTTA 1165
ACTGAAGAAA ACCTGGAGGC TAGCCTTGGG GGCAGGAGGA GGCATCCTTT GAGCATCCCC 1225
ACCCCACCCA ACTTCAGCCT CGTGACACGT GGGAACAGGG TTGGGCAGAG GTGTGAACA  1285
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FIG. 4B-3

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GCACAAGGCC AGAGACCACG GCATGCCACT TGGGTGCTGC TCACTGGTCA GCTGTGTGTC 1345
TTACACAGAG GCCGAGTGGG CAACACTGCC ATCTGATTCA GAATGGGCAT GCCCTGTCCT 1405
CTGTACCTCT TGTTCAGTGT CCTGGTTTCT CTTCCACCTT GGTGATAGGA TGGCTGGCAG 1465
GAAGGCCCCA TGGAAGGTGC TGCTTGATTA GGGGATAGTC GATGGCATCT CTCAGCAGTC 1525
CTCAGGGTCT GTTTGGTAGA GGGTGGTTTC GTCGACAAAA GCCAACATGG AATCAGGCCA 1585
CTTTTGGGGC GCAAAGACTC AGACTTTGGG GACGGGTTCC CTCCTCCTTC ACTTTGGATC 1645
TTGGCCCCTC TCTGGTCATC TTCCCCTGCC CTTGGGCTCC CCAGGATACT CAGCCCCTGAC 1705
TCCCATGGGG TTGGGAATAT TCCTTAAGAC TGGCTGACTG CAAAGGTCAC CGATGGAGAA 1765
ACATCCCTGT GCTACAGAAT TGGGGGTGGG ACAGCTGAGG GGGCAGGCGG CTCTTTCCTG 1825
ATAGTTGATG ACAAGCCCTG AGAATGCCAT CTGCTGGCTC CACTCACACG GGCTCAACTG 1885
TCCTGGGTGA TAGTGACTTG CCAGGCCACA GGCTGCAGGT CACAGACAGA GCAGGCAAGC 1945
AGCCTTGCAA CTGCAGATTA CTTAGGGAGA AGCATCCTAG CCCCAGCTAA CTTTGGACAG 2005
TCAGCATATG TCCCTGCCAT CCCTAGACAT CTCCAGTCAG CTGGTATCAC AGCCAGTGGT 2065
TCAGACAGGT TTGAATGCTC ATGTGGCAGG GGGCCCGGTA CCCAGCTTTT GTTCCCTTTA 2125
GTGAGGGTTA ATTGCGCGCT TGGGCTAATC ATGGTCATAG CTGTTGGGCG TTGCTGGGCGT 2185
TTTTCCATAG GCTCCGCCCC CTGACGAGAT CACAAAAATC GACGCTCAAG TCAGAGGTGG 2245
CGAAACCGAC AGACTATAAG ATACCAGGC                                   2274
```

FIG. 4B-4

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) : G01N 33/543; C12Q 1/68; C07K 15/00
US CL   : 436/518; 435/6; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   436/518, 536; 435/6, 7.2, 7.21; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Nature, Vol. 362, issued 04 March 1993, Nishimoto et al., "Alzheimer amyloid protein precursor complexes with brain GTP-binding protein Go," pages 75-79, see entire document.	1-20, 27-29

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be part of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 APRIL 1994	25 APR 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DONNA C. WORTMAN
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

ational application No.

PCT/US94/01712

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

    I.   Claims 1-20, 27-29, drawn to a composition and a method of use, Class 436, Subclass 518, and Class 530, subclass 350.

    II.   Claims 21-26, drawn to a treatment method, Class 512, Subclass 12.

          Groups I and II do not share a common special technical feature as represented in PCT Rule 13.2 because they are drawn to completely different methods requiring different process steps for completion. Note that PCT Rule 13.2 does not provide for multiple methods within a single inventive concept.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-20, 27-29

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                      ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: LIPOPOLYSACCHARIDE-BINDING AND NEUTRALIZING PEPTIDES

(57) Abstract

    The present invention relates to substances which bind with high affinity to endotoxin (lipopolysaccharide [LPS]), and which are useful for the prevention or treatment of, for example, Gram-negative and Gram-positive bacterial sepsis, and for the treatment of bacterial and fungal infections as well as for neutralizing effects associated with heparin. The substances are LPS-binding peptides comprising an LPS-binding domain. In addition, the invention relates to DNA sequences encoding said peptides, recombinant microorganisms containing said DNA, pharmaceutical compositions containing the peptides of the invention, and diagnostic kits. The invention also encompasses methods for the detection and removal of bacterial LPS from solutions.

Lipopolysaccharide-Binding and Neutralizing Peptides

The present invention relates to substances which bind with high affinity to endotoxin (lipopolysaccharide [LPS]), and which are useful for the prevention or treatment of a variety of conditions and diseases, such as of Gram-negative and Gram-positive bacterial sepsis, or bacterial or fungal infections. Furthermore, said substances may be used for neutralizing effects associated with heparin. The substances are LPS-binding peptides comprising an LPS-binding domain. The invention also encompasses methods for the detection and removal of bacterial LPS from solutions.

In humans, LPS released during infection by Gram-negative bacteria can cause the severe pathological changes associated with septic shock (Duma, *Am. J. Med.* 78 (1985) 154-163; Glauser et al., *Lancet* 338 (1991), 732-736). In the United States, septic shock is responsible for between 100,000 and 300,000 deaths annually (Ziegler et al., *N. Eng. J. Med.* 324 (1991), 429-436) and in Germany for between 70,000 and 100,000. Although a variety of agents have been evaluated for neutralizing LPS or enhancing its clearance *in vivo*, there remains no specific treatment for Gram-negative bacterial sepsis.

LPS is a glycolipid that is ubiquitous in the outer membrane of Gram-negative bacteria (Raetz, *Annu. Rev. Biochem.* 59 (1990), 129-170). LPS consists of an oligosaccharide and a lipid portion and is characterized by an overall negative charge, stability to heat, and high molecular weight. While the chemical structure of most LPS molecules is complex and diverse, a common feature is the lipid A region. Lipid A, the membrane anchor of LPS, consists of a central phosphodisaccharide unit that is attached to up to seven fatty acid chains. Most of the biological activities of LPS reside in the lipid A portion (Galanos et al., *Eur. J. Biochem.* 145, (1985), 1-5).

2

Septic shock is a complex condition which arises from a
cascade of molecular and cellular events following infection
by microorganisms, predominant among which are Gram-negative
bacteria. The onset of shock arises from the interaction of
LPS or lipid A with membrane-bound receptors on macrophages
and blood monocytes (Couturier et al., *J. Immun.* 147 (1991),
1899-1904) or various serum proteins, such as the septins
(Wright et al., *J. Exp. Med.* 176 (1992), 719-727). These
interactions lead to an increase in the levels of pro-
inflammatory mediators such as tumor necrosis factor, IL-1,
IL-6, and interferon-c. Endothelial cells are also stimulated
to produce factors which attract neutrophils. Release of
enzymes and other factors by activated neutrophils causes
damage to local vasculature which can lead rapidly to death.

One approach to the treatment of sepsis is the use of
substances which bind to LPS and neutralize its toxic effects
in vivo. Although there are numerous proteins which bind LPS,
the number of substances which effectively neutralize LPS *in
vivo* are very few. A number of such substances have been
identified, including polymyxins (Morrison et al.,
Immunochem. 13 (1976), 813-818), polymyxin-derived peptides
(Rustici et al., *Science* 259 (1993), 361-365), polyclonal
(Ziegler et al., *N. Eng. J. Med.* 307 (1982), 1225-1230) and
monoclonal (Ziegler et al. (1991), loc. cit.) antibodies,
bactericidal/permeability-increasing protein (BPI) (Marra et
al., *J. Immun.* 148 (1992), 532-537), lipopolysaccharide
binding protein (LBP) (Schuhmann et al., *Science* 249 (1990),
1429-1431), and Limulus anti-LPS factor (LALF) (Akategawa et
al., *J. Biol. Chem.* 261 (1986), 7357-7365, Muta et al., *J.
Biochem.* 101 (1987), 1321-1330).

The simplest molecules that bind to the lipid A portion of
LPS with high affinity are the polymyxin antibiotics; these
are positively charged amphipathic cyclic oligopeptides
attached to a lipid tail. Although polymyxins bind to

LPS/lipid A with high affinity, they suffer the drawback from
a therapeutic stand-point of having unacceptably high
toxicity (Craig et al., *Infect. Immun.* 10 (1974), 287-292).
The LPS-binding monoclonal antibodies HA-1A and E5 both
failed to demonstrate positive clinical effects for the
treatment of Gram-negative septic shock. One of the main
problems associated with these antibodies is non-specificity;
for example, HA-1A binds tightly to numerous hydrophobic
structures apart from lipid A (see, for example, Baumgartner
et al., *J. Exp. Med.* 171 (1990), 889-896). The human proteins
BPI and LBP are both being investigated for the treatment of
Gram-negative sepsis (Marra et al., loc. cit.; Ulevitch et
al. (1986), WP 86/06279). BPI, which is stored in specific
granules of polymorphonuclear cells, kills Gram-negative
bacteria by binding to membrane-bound LPS and disrupting the
permeability barrier. LBP is a mammalian serum protein which
also binds very tightly to LPS. Although LBP shares sequence
homology with BPI (Schuhmann et al., loc. cit.), it is not
directly cytotoxic to Gram-negative bacteria and its precise
function is obscure. Most recently, LALF has been
investigated for use in sepsis (Warren et al., *Infect. Immun.*
60 (1992), 2506-2513; Wainwright et al. (1992) WO 92/20715).
This protein is almost certain to suffer the disadvantages
associated with other foreign proteins for human therapy; it
is immunogenic and has only a short half-life in circulation.
These factors will reduce its clinical potential. None of
these substances have been proven to be effective for the
treatment of the serious conditions associated with Gram-
negative infection.

Thus, the technical problem underlying the present invention
is to provide substances which bind LPS released by Gram-
negative bacteria, neutralize its toxic effects, and exhibit
no toxicity.

The solution to the above technical problem was achieved by
providing substances which relate to peptides which bind

4

tightly to LPS, and therefore have utility in the diagnosis
and treatment of Gram-negative and other septic conditions.

Thus, the present invention relates to LPS-binding peptides
comprising an LPS-binding domain comprising at least:

(a) the amino acid sequence 1-2-3-4-5-6-7-8, wherein the
    numbers represent any of the following amino acids:

       1 = a polar or positively charged amino acid,
    preferably
    C, H, K, N, Q, R, S, T, W, or Y;

       2 = a hydrophobic amino acid, preferably
    A, F, H, I, L, M, V, or W;

       3 = a basic amino acid, preferably H, K, or R;

       4 = a hydrophobic or positively charged amino acid,
    preferably
    A, F, H, I, K, L, M, R, V, or W;

       5 = a hydrophobic, polar, or positively charged amino
    acid, preferably
    A, C, F, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

       6 = a positively charged amino acid, preferably K or
    R;

       7 = A hydrophobic, polar, or positively charged amino
    acid, preferably
    A, C, F, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

       8 = a hydrophobic or positively charged amino acid,
    preferably
    A, F, H, I, K, L, M, R, V, or W;

(b) a corresponding inverse amino acid sequence; or

(c) a variation of said amino acid sequence (a) or (b) capable
    of effectively binding to LPS.

The peptides of the present invention _effectively bind to
LPS_, i.e. they interact specifically with LPS with an
association constant greater than 10^5 M^{-1}. In this context,
an LPS-binding peptide is a chain of amino acids linked to
each other by peptide bonds. An LPS-binding domain is the

shortest possible chain of amino acids within an LPS-binding peptide which effectively binds to LPS.

All peptide structures disclosed use the single letter code for amino acids.

The figures show:

Figure 1: A schematic diagram of the LALF loop indicating the direction of side-chains and the putative locations of the corresponding residues in LBP and BPI. The three letters at each position correspond to the amino acid residues in LALF, LBP and BPI, respectively. Solid bonds/dashed bonds indicate side chains pointing out of/into the plane of the diagram.

Figure 2 shows the peptides which are used to define a minimum LPS-binding domain in LALF. The two cysteine residues are linked by a disulphide bond.

Figure 3 shows the peptides which are used to define a minimum LPS-binding domain in LBP. The two cysteine residues are linked by a disulphide bond.

Figure 4 shows the peptides which are used to define the LPS-binding domain in BPI. The two cysteine residues are linked by a disulphide bond.

Figure 5 shows the aligned sequences of LALF, LBP and BPI in which the LBP and the BPI sequences are listed from the C-terminus to the N-terminus while the LALF sequence is written from the N-terminus to the C-terminus.

Figure 6 shows the peptides which are used to define the LPS-binding activity in the inverse orientation. The two cysteine residues are linked by a disulphide bond.

6

Figure 7 shows a list of the peptides in which point mutations are introduced into peptides resembling the minimal LPS binding domain of LBP. The two cysteine residues are linked by a disulphide bond.

Figure 8 shows ELISA data for the binding of LALF-derived peptides to lipid A. The four biotinylated peptides are allowed to bind to immobilized lipid A, and bound peptide is detected using a streptavidin-alkaline phosphatase conjugate. For more details, see Example 1.

Figure 9 shows ELISA lipid A binding data for peptides derived from LBP, BPI, and the generalized motif described here in comparison with the LALF-derived peptide. The biotinylated peptides are allowed to bind to immobilized lipid A, and bound peptide is detected using a streptavidin-alkaline phosphatase conjugate. For more details, see Example 1.

Figure 10 shows ELISA data for the binding of the LALF-derived peptide LALF-14 to different types of lipopolysaccharide. The biotinylated peptide is allowed to bind to immobilized lipid A or LPS, and bound peptide is detected using a streptavidin-alkaline phosphatase conjugate. For more details, see Example 2.

Figure 11 shows the ELISA data which reveals the difference between cyclic and linear peptides in binding to lipid A. The biotinylated peptides are allowed to bind to immobilized lipid A, and bound peptide is detected using a streptavidin-alkaline phosphatase conjugate. For more details, see Example 3.

Figure 12 shows ELISA data for the binding of fluorescein-labelled LPS to biotinylated peptides derived from LALF, LBP, BPI and the generalized motif described here, which are

7

immobilized on the ELISA plate through interaction with streptavidin. For more details, see Example 4.

Figure 13 shows ELISA data for the competition between LALF and LALF-derived peptides for binding to lipid A. Increasing concentrations of peptide are allowed to compete with a fixed concentration of LALF for binding to immobilized lipid A. Bound LALF is detected using an anti-rabbit antibody conjugated to alkaline phosphatase. For more details, see Example 5.

Figure 14 shows ELISA data for the competition between LALF and various peptides for binding to immobilized lipid A at a single peptide concentration. The extent to which the peptide inhibits LALF binding (measured via an anti-rabbit antibody) to lipid A is determined at 100 μg/ml of peptide. For more details, see Example 5.

Figure 15 shows ELISA data for the competition between LBP and various peptides for binding to immobilized lipid A at a single peptide concentration. The extent to which the peptide inhibits LBP binding (measured via an anti-rabbit antibody) to lipid A is determined at 100 μg/ml of peptide. For more details, see Example 6.

Figure 16 shows ELISA data for the competition between the peptide LBP-14 and various peptides and proteins for binding to immobilized lipid A at a single peptide concentration. The extent to which the biotinylated LBP-14 inhibits peptide or protein binding (measured using a streptavidin-alkaline phosphatase conjugate) to lipid A is determined at 1 μg/ml and 100 μg/ml of peptide. For more details, see Example 7.

Figure 17 shows ELISA data which illustrates the effect of increasing concentrations of serum on the binding of peptides to immobilized lipid A. Biotinylated peptides are detected

8

using a streptavidin-alkaline phosphatase conjugate. For more
details, see Example 8.

Figure 18 shows ELISA data which illustrates the effect of
increasing concentrations of serum on the ability of peptides
to compete with LALF for binding to immobilized lipid A. The
extent to which the peptide inhibits LALF binding (measured
via an anti-rabbit antibody) to lipid A is determined at 100
µg/ml of peptide of 0% and 10% fetal calf serum. For more
details, see Example 9.

Figure 19 shows data for the inhibition by peptides of the
Limulus amebocyte lysate gelling and chromogenic assays. The
peptides are tested either for their ability to inhibit LPS-
mediated gelling of Limulus amebocyte lysates or to inhibit
the LPS-colour reaction in the chromogenic Limulus amebocyte
lysate assay. For more details, see Example 10.

Figure 20 shows data for the inhibition, by a fixed
concentration of four different peptides, of the LPS-mediated
release of tumour necrosis factor by monocytes. TNF is
detected using a commercial ELISA kit. For more details, see
Example 11.

It has surprisingly been found by the inventors that the
crystal structure of LALF reveals a novel but simple tertiary
fold which has a striking shape and amphipathicity. A
surface-extended loop in the LALF structure (loop of LALF or
LALF-loop) has similar features to polymyxin B by being
positively charged and amphipathic and having several exposed
hydrophobic and aromatic residues. Furthermore, the loop of
LALF is distinguished by an alternating series of positively
charged and hydrophobic/aromatic residues that, by virtue of
the extended ß-conformation, point in opposite directions,
and a single pair of positive charges, that, because of the
ß-turn conformation, point in the same direction and maintain

the amphipathicity. The loop contains no negatively charged amino acids.

A similar amphipathic loop exists in three other proteins which bind LPS: rabbit and human lipopolysaccharide-binding protein (LBP) and human bactericidal/permeability-increasing protein (BPI) (Figure 1). Inspection of the LBP and BPI sequences reveals a similar pattern of alternating residues that could produce an amphipathic loop; the 19 residue stretch contains six basic and no acidic amino acids. Near the top of the loop, one amphipathic pair of residues (Ser/Arg96 and Phe97) is reversed, but it is possible that a different conformation of the hairpin turn would maintain the amphipathicity of the loop in BPI and LBP.

The deduced sequence and structural homology within this set of LPS-binding proteins led to the design of the peptides which are the subject of the present invention. All of the LPS-binding peptides encompassed by the present invention can be prepared using standard methods of peptide synthesis, as described by, for example, S.D.H. Kent (*Ann. Rev. Biochem.* 57, (1988), 957), which is apparent to anyone skilled in the art. Alternatively, the peptides can be synthesized biologically using a recombinant microorganism which has been genetically engineered to contain DNA sequences encoding the peptides (Sambrook et al., *Molecular Cloning, A Laboratory Manual*, CSH Press (1989)).

In another embodiment, the present invention relates to peptides having an <u>inverse</u> amino acid sequence derived from an LPS-binding peptide and also effectively binding to LPS. Thus, the present invention also encompasses such peptides. In this context, an inverse amino acid sequence is a chain of amino acids which, when read from the N-terminus to the C-terminus, has the same sequence as the parent peptide when read from the C-terminus to the N-terminus. There exists homology between LBP/BPI sequences from amino acids 90 to 97 and the LALF sequence from residues 38 to 45 when the LBP/BPI sequence is read from the C-terminus to the N-terminus

(Figure 5). On this basis, "inverse" peptides were
synthesized and tested for their ability to bind LPS.
Particularly preferred are the inverse peptides shown in
Figure 6 which effectively bind to LPS, as shown by ELISA.

In still another embodiment, the present invention relates to
peptides which share a common structural motif with the above
peptides and are also able to bind LPS. Thus, the present
invention also provides for an LPS-binding peptide comprising
an LPS-binding domain in which the amino acid sequence
includes a variation. In this context, a variation of an
amino acid sequence refers to any changes in the sequence
that are introduced either by an insertion or a deletion of
one or more amino acids. Particularly preferred are the
peptides shown in Figure 7, in which mutations have been
introduced in the parent peptide structure. These altered
peptides both bind to LPS, as shown by ELISA.

In a preferred embodiment, the present invention provides
LPS-binding peptides, wherein the amino acid sequence (a) is
any combination of the amino acids seen in the LPS-binding
loops of LALF, LBP, or BPI, namely:

        1 = T or R or K
        2 = F or W
        3 = R or K
        4 = R or V or A
        5 = L or R or Q
        6 = K
        7 = W or S or R
        8 = K or F

In a further preferred embodiment, the present invention
provides peptides of the general motif outlined above, with
the additional feature that the N-terminus is extended by two
or more amino acids denoted -2 and -1, in which amino acid
-2, which is the new N-terminus, is taken from the set R, K,
H, N and Q, and amino acid -1 is any amino acid.

In a further preferred embodiment, the present invention provides peptides of the general motif outlined above with the additional feature that the C-terminus is extended by addition of cysteine, and the N-terminus is extended by two or more amino acids denoted -2 and -1 in which the amino acid -2, which is the new N-terminus, is taken from the set R, K, H, N and Q, and amino acid -1 is cysteine, the two cysteines being linked by a disulphide bond.

In a more preferred embodiment, the LPS-binding peptides have the amino acid sequence TFRRLKWK, RWKVRKSFFKLQ, or KWKAQKRFLKMS.

In a further preferred embodiment, the present invention provides linear peptides which are able to bind LPS effectively. Particularly preferred are peptides derived from LALF. Disulfide-constrained circular peptides *(see below)* spanning amino acids 31 to 52 and amino acids 38 to 45 of LALF are incubated with DTT to reduce the disulfide bond. The peptide spanning amino acids 31 to 52 binds effectively to lipid A as determined by ELISA. The peptide spanning residues 38 to 45 interacts only weakly with lipid A, as determined by ELISA.

In a still further preferred embodiment, the present invention provides for peptides which effectively bind to LPS and are constrained to adopt a circular conformation by an intramolecular bridge. In this context, the circular conformation can be brought about by any one of a number of intramolecular bridges. Preferably, the peptide may incorporate two cysteine residues between which a disulfide bond is formed by an oxidation reaction. Alternatively, the two cysteines may be linked through a homo-bifunctional cross-linking reagent, such as a bis-maleimide.
Particularly preferred are the cyclic peptides comprising amino acids 31 to 52 and amino acids 38 to 45 of LALF, each stabilized by a disulfide bond formed between two cysteine

12

residues, producing a cyclic conformation (Figure 2). Both
bind lipid A and, additionally, two different types of LPS-
molecules, E. coli J5-LPS and E. coli EH 100 LPS, as
determined by ELISA. This defines a minimal LPS-binding
domain to be the peptide spanning amino acids 38 to 45 of
LALF.
Peptides derived from LBP according to the alignment as shown
in Figure 1, which are constrained to adopt a cyclic
conformation by means of a disulfide bond formed between two
unique cysteine residues, are also able to bind LPS, as shown
by ELISA. Thus, particularly preferred are also the peptides
spanning amino acids 90 to 101 and amino acids 90 to 97 of
LBP (Figure 3). Conversely, the peptide spanning amino acids
92 to 99 of LBP does not bind LPS. This defines a second
minimal LPS binding domain to be the peptide spanning amino
acids 90 to 97 of LBP. Furthermore, the fact that the peptide
ranging from amino acids 92 to 99 does not bind lipid A
although it is positively charged and amphipathic indicates
that these features alone are not sufficient to provide an
LPS-binding motif, further illustrating the novelty of the
present invention.
Peptides derived from BPI according to the alignment shown in
Figure 1, which are constrained to adopt a cyclic
conformation by means of a disulfide bond between two unique
cysteine residues, are also able to bind LPS as shown by
ELISA. Particularly preferred are the peptides spanning amino
acids 90 to 97 and amino acids 90 to 101 of BPI (Figure 4).
This defines a third minimal LPS-binding domain to be the
peptide spanning amino acids 90 to 97 of BPI.

In a further preferred embodiment, the present invention
provides a detectably-labeled peptide which can be used in an
assay for the determination of LPS in a biological sample. In
this context, a detectably-labeled peptide is a peptide which
is covalently linked to a substance which can readily be
detected. Most commonly, the label is an enzyme, fluorescent
substance, or radionuclide. By way of example, the peptide

may be linked to an enzyme such as ß-galactosidase, peroxidase, or alkaline phosphatase, which, in the presence of an appropriate substrate, can lead to the generation of a colored or fluorescent product which is readily detected. Alternatively, the peptide may be linked directly to a fluorescent substrate, such as fluorescein, rhodamine, or auramine for the purposes of detection. Commonly used labelling radionuclides include ^{14}C, ^{131}I, ^{3}H, ^{125}I, and ^{35}S. Many variations on labelling configurations can be imagined. For example, the peptide may be linked through an intermediary substance, such as biotin, to another substance, such as streptavidin, which is itself linked to a substance which enables detection according to conventional methods.

In a preferred embodiment, the present invention provides a set of LPS-binding peptides each comprising one or more LPS-binding domains.

The present invention also includes DNA sequences encoding the peptides of the present invention, as well as vectors, such as plasmids, phagemids, and cosmids containing these DNA sequences.

Additionally, the present invention encompasses microorganisms such as viruses, bacteria and yeast which have been transformed with these vectors. The DNA sequences provided herein are most readily obtained using standard methods of automated DNA synthesis, but can also be obtained by conventional molecular cloning. For example, those DNA sequences derived from the naturally-occurring proteins LBP, BPI, or LALF can be obtained in a form suitable for cloning by use of the polymerase chain reaction, as will be apparent to anyone skilled in the art (Sambrook et al., loc. cit.).

Furthermore, the present invention relates to a method for the production of an LPS-binding peptide according to the invention, comprising culturing a microorganism transformed

14

with a recombinant vector comprising a DNA encoding the peptide of the invention, and recovering said peptide or a fusion protein containing it from the medium.

In a preferred embodiment, the present invention provides a pharmaceutical composition comprising effective amounts of any of the peptides described above in combination with a pharmaceutically acceptable carrier and/or diluent. The pharmaceutical composition can be used for the treatment of a variety of conditions related to the release of LPS, especially Gram-negative sepsis. In this context, the term sepsis refers to the morbid conditions induced by a toxin, the introduction or accumulation of which is most commonly caused by infection or trauma. The initial symptoms of sepsis typically include chills, profuse sweat, irregularly remittent fever, prostration and the like, followed by persistent fever, hypotension leading to shock, neutropenia, leukopenia, disseminated intravascular coagulation, adult respiratory distress syndrome and multiple organ failure.

The peptides which are the subject of the present invention are similar in structure to a number of peptides which derive from BPI, which have been the subject of investigation for their therapeutic properties (see, for example, Little et al., *J. Biol. Chem.* **269** (1994) 1865-1872). In addition to their endotoxin binding and neutralization capabilities by comparison with the BPI-derived peptides, the peptides which are described here may be expected to show bactericidal and heparin binding activity. Accordingly, the present invention furthermore provides pharmaceutical compositions which can be used to kill bacteria (both Gram-negative and Gram-positive) and fungi as well as pharmaceutical compositions which possess the ability to neutralize properties associated with heparin, such as anticoagulation, angiogenesis, and growth factor-induced tumour and endothelial cell proliferation.

In a further preferred embodiment, the present invention also encompasses a diagnostic kit. Such a kit would comprise at least a peptide or a labelled peptide as set out above and would consist additionally of the reagents and materials necessary to carry out a standard competition or sandwich assay. Said diagnostic kit can be used for the determination of LPS or for the diagnosis of septic conditions.

In a still further preferred embodiment, the present invention provides the peptides disclosed herein immobilized on a solid support. Most conveniently, the peptide is covalently linked to a solid support such as cellulose, agarose, polyacrylamide, etc, which is modified so as to bear a reactive functionality such as an imidate, activated ester, activated disulfide, epoxide, etc. The peptide can be linked by any one of a number of methods which are commonly used in protein chemistry. By way of example, an N-terminal or C-terminal cysteine can readily be introduced into the peptide during synthesis. The thiol function of this cysteine residue can be used to link the peptide to a solid support which has been derivatized to bear a maleimide group. The present invention also provides a method of removing LPS from solutions, whereby said solution is passed over the immobilized peptides of the invention. This method is of particular interest in the purification of pharmaceutical preparations, LPS contamination of which is a frequently-occurring problem.

Now that the invention has been generally described, it will be illustrated by the following specific examples, which are provided for the purpose of illustration only and are not intended to limit the scope of the invention.

Example 1: Binding of peptides to lipid A

Peptides are synthesized using standard Fmoc chemistry on an ABIMED AMS 422 synthesizer. The cleaved peptides are oxidized

16

overnight in DMSO at room temperature, purified on a C-18
reversed phase column with a gradient of acetonitrile/0.1%
trifluoroacetic acid, and characterized by mass spectroscopy.

To confirm the hypothesis that the loop sequence of LALF
binds lipid A and LPS, peptides comprising parts of the LALF
loop are synthesized, ranging in length from 8 to 12 amino
acids. The peptides comprising amino acids 36 to 47 (LALF-
14), and amino acids 38 to 45 (LALF-10) of LALF, are
stabilized by a disulfide bond through the introduction of
cysteine residues at the N- and C- termini of the peptides,
thus adopting a cyclic conformation. The peptide comprising
amino acids 36 to 45 (LALF-11), in which amino acid 37 is
replaced by a cysteine is also cyclized following
introduction of a second cysteine residue at the C-terminus
of the peptide. All peptides are labelled at the N-terminus
with biotin.

The lipid A-binding activities of the peptides are shown in
an ELISA format as follows. An ELISA plate (NUNC, Polysorp
96U) is coated for 90 minutes at 37°C with 100 μl lipid A in
PBS (0.25-1 μg/ml). All further steps are done at room
temperature. After washing and blocking (10 min.) with
PBS/0.1% Tween, the solid phase is incubated for 1 hour with
100 μl of increasing amounts (0.1 μg/ml - 10 μg/ml) of
purified synthetic peptides (dissolved in PBST) labelled at
the N-terminus with biotin. After washing with PBS/0.1%
Tween, the bound peptides are incubated for 45 min. with 100
μl streptavidin conjugated to alkaline phosphatase
(Boehringer Mannheim GmbH; 1:10000 diluted in PBS/0.1%
Tween), followed again by washing with PBS/0.1% Tween and
100mM Tris pH 9.5. p-Nitrophenylphosphate (2 mg/ml in 100mM
Tris pH 9.5) is used as a substrate for alkaline phosphatase.
The ELISA is read at 405nm.

Increasing amounts of peptide are added to immobilized lipid
A. LALF-14, LALF-11 and LALF-10 bind lipid A with high

activity (see Figure 8). The cyclic peptide comprising the inverted sequence from amino acids 38-45, RETL-10, binds lipid A only slightly above background level, which is set by the control peptide. This result indicates that, although RETL-10 is positively charged and amphipathic, these features alone are not sufficient to provide high affinity LPS-binding, illustrating the novelty of the present invention.

The peptides derived from LBP comprising amino acids 90 to 101 (LBP-14), amino acids 92 to 99 (LBP-10-1) and amino acids 90 to 97 (LBP-10-2), the peptide derived from BPI comprising amino acids 90 to 101 (BPI-14), and two peptides comprising an amino acid sequence randomly chosen from the motif defined herein (MS-21 (Biotin-Z-K-C-F-T-R-R-A-K-W-R-C (Z = ß-alanine)), MS-22 (Biotin-Z-C-K-W-K-I-R-K-F-S-C-N (Z = ß-alanine))) are stabilized by formation of a disulfide bond formed by the oxidation of cysteines introduced at the N- and C- termini of the peptides, giving cyclic peptides. Each is labelled at the N-terminus with biotin.

The lipid A-binding activity of the peptides is tested in an ELISA format as described above, in which increasing amounts of peptide is added to immobilized lipid A (see Figure 9). LALF-14 is used as a control for high activity binding and the irrelevant peptide Nor (G-A-T-P-E-D-L-N-T-L) to determine background binding. Of the new peptides based on LBP, LBP-14 binds lipid A as well as LALF-14, LBP-10-1 only in its highest concentration (10 µg/ml) binds lipid A above background, while LBP-10-2 binds lipid A only slightly above background. The peptide based on BPI, BPI-14, binds lipid A above background, but significantly weaker than LALF-14 and LBP-14. Of the peptides based on the LPS binding motif, MS-21 and MS-22, MS-21 binds lipid A weaker than LALF-14 and LBP-14, but slightly better than BPI-14. MS-22 binds lipid A clearly above background level and with a similar activity to LBP-10-1, but weaker than MS-21.

Example 2: Binding of LALF-14 to lipid A and LPS

The cyclic peptide comprising amino acids 36 to 47 of LALF
(LALF-14) is tested for binding different kinds of
lipopolysaccharides in comparison with lipid A. in an ELISA
format in which increasing amounts of peptide are added to
immobilized lipid A and LPS. The forms of lipopolysaccharide
used are E.coli Re F515; Klebsiella p.; Salmonella e.;
Shigella f.; and E.coli 0127:B8. LALF-14 is able to bind to
all species of lipopolysaccharide above background, but with
different activities (see Figure 10). Thus, LALF-14 binds
Salmonella e. as well as it binds to lipid A, E.coli Re F515
and Shigella f. with less activity than lipid A but with
higher activity than Klebsiella p. and E.coli 0127:B8.

Example 3: Importance of conformation for peptide-lipid A binding

To investigate the importance of the constrained conformation
for high affinity peptide-lipid A binding, binding activity
of cyclic and the corresponding linear peptides is compared.
This is achieved (i) by reducing peptides LALF-14, LBP-14 and
BPI-14 with DTT to destroy the disulphide bond and (ii) by
synthesizing a linear peptide comprising amino acids 36 to 45
(LL-10) for comparison with LALF-11. The lipid A-binding
activity of the peptides is tested in an ELISA format (vide
infra) in which increasing amounts of peptide are added to
immobilized lipid A. In all cases, the oxidized peptides
LALF-14, LBP-14 and BPI-14 display higher lipid A binding
activity compared with the reduced peptides, LALF-14/DTT,
LBP-14/DTT and BPI-14/DTT, demonstrating that a constrained
cyclic form is superior to a linear conformation for high
lipid A binding activity (see Figure 11). This result is
confirmed by the observation that the lipid A binding
activity of the cyclic peptide LALF-11 is higher than that of
its linear counterpart LL-10.

19

Example 4: Binding of fluorescein-labelled LPS to biotinylated peptides

To investigate the potential use of multimeric peptides for increasing the binding to LPS, individual peptides are coated on an ELISA plate to high surface concentrations. Immobilization is performed by first coating the plate with streptavidin and then binding the peptides via their N-terminal biotin groups. Thus, 100 μl streptavidin (Boehringer Mannheim GmbH; 10 μg/ml in PBS) is used for coating followed by washing, blocking (10 min.) with PBS/0.1% Tween and a 30 min. incubation with 100 μl of purified synthetic peptides (1 μg/ml or 10 μg/ml in PBST) labelled at the N-terminus with biotin. After washing with PBS/0.1% Tween the peptides are incubated for 1 hr with FITC-labelled LPS (SIGMA; Salmonella enteriditis; 0.5 μg/ml in PBS/0.1% Tween). After washing with PBS/0.1% Tween, the ELISA plate is incubated with 100 μl of an anti-FITC antibody conjugated to alkaline phosphatase (SIGMA; 1:2500 diluted in PBS/0.1% Tween) and washed with PBS/0.1% Tween and 100mM Tris pH 9.5. p-Nitrophenyl-phosphate (2 mg/ml in 100mM Tris pH 9.5) is used as a substrate for alkaline phosphatase. The ELISA is read at 405nm. All steps are done at room temperature.

As expected, LALF-14, LALF-11, LALF-10, LBP-14 and MS-21 showed the highest binding affinity to LPS (see Figure 12). Peptides which bind lipid A slightly above background level as determined in the alternative ELISA format, such as RETL-10, LBP-10-1 or LBP-10-2, show binding in this format clearly above (2-3 fold) background. This indicates that oligomeric binding sites for LPS can provide higher LPS binding activity.

Example 5: Competition of LALF: lipid A binding by peptides

To investigate the specificity of peptides in binding lipid A and LPS, competition experiments with known endotoxin binding

proteins such as LALF and LBP and the antibiotic polymyxin B are carried out. Thus an ELISA plate (NUNC, Polysorp 96U) is coated for 90 min. at 37°C with 100 μl lipid A in PBS (0.25 - 0.3 μg/ml) or lipopolysaccharide in PBS (0.25 - 0.3 μg/ml). All further steps are done at room temperature. After washing and blocking (10 min.) with PBS/0.1% Tween, the solid phase is incubated for 1 hr with 100 μl of increasing amounts (0.01 μg/ml - 100 μg/ml in PBS/0.1% Tween) of unlabelled or biotin-labelled peptides mixed with an endotoxin binding protein (LALF or LBP; 0.2 μg/ml in PBS/0.1% Tween). After washing with PBS/0.1% Tween, the ELISA plate is incubated for 45 min. with 100 μl of a rabbit antiserum against LALF or LBP, respectively. After washing with PBS/0.1% Tween the ELISA plate is incubated with 100 μl anti-rabbit antibody conjugated to alkaline phosphatase (SIGMA; 1:10000 diluted in PBS/0.1% Tween) and washed with PBS/0.1% Tween and 100mM Tris pH 9.5. p-Nitrophenyl-phosphate (2 mg/ml in 100mM Tris pH 9.5) is used as a substrate for alkaline phosphatase. The ELISA is read at 405nm.

If assaying for LALF-lipid A binding, increasing amounts of peptide should lead to a decrease of detectable LALF bound to lipid A (see Figure 13). Polymyxin B is used as a positive control, and at sufficiently high concentration is able to displace LALF almost completely from immobilized lipid A. A control peptide and lysozyme, also known to bind lipid A and LPS, are not able to compete with LALF. Of the peptides based on the LALF sequence, LALF14, LALF-11 and L-10 are as capable as polymyxin B in competing the LALF/lipid A binding, indicating a similar LPS binding activity as polymyxin B, while RETL-10 only weakly competes with LALF.

At 100 μg/ml, LALF-14, and polymyxin B compete with LALF for lipid A binding up to 95%, while a control peptide or lysozyme compete to 10% only. Of the peptides based on LBP, H-14 competes with LALF for lipid A binding as effectively as LALF-14 or polymyxin B, while only at the highest

21

concentrations do LBP-10-1 and H-10 inhibit the LALF lipid A binding. BPI-14 competes to a lesser extent compared with LALF-14, H-14 or polymyxin B. Of peptides based on the generalized lipid A binding motif, MS-21 competes with LALF for binding to lipid A to almost 80% while MS-22 is similar to BPI-14. PolP-1 (I-K-T-K-K-F-L-K-K-T), a peptide based on polymyxin B and shown to bind lipopolysaccharide and neutralize its toxicity (Rustici et al.. *Science* 259 (1993), 361-365), hardly competes with LALF for lipid A binding.

These data are presented in bar graph form in Figure 14 for a given concentration of peptide.

Example 6: Competition of LBP : lipid A binding by peptides

The ability of the peptides to compete with LBP for binding to LPS is determined in the same way as in Example 5, except that LALF is replaced with LBP (see Figure 15). The result of this experiment is, with one exception, very similar to the result in Figure 14. As shown in Figure 15, LALF-14, L-10 and H-14 are the most potent inhibitors of the LBP-lipid A binding. In contrast to the result observed with LALF, in this case polymyxin B is not able to compete with LBP for binding to lipid A.

Example 7: Competition of LBP-14: lipid A binding by peptides and proteins

An ELISA plate (NUNC, Polysorp 96U) is coated for 90 min. at 37°C with 100 μl lipid A in PBS (0.2 - 0.3 μg/ml). All further steps are done at room temperature. After washing and blocking (10 min.) with PBS/0.1% Tween, the solid phase is incubated for 1 hr with 100 μl of increasing amounts (0.01 μg/ml - 10 μg/ml) of unlabelled peptides or proteins mixed with a biotin-labelled peptide (1 μg/ml in PBST/0.1% Tween). After washing with PBS/0.1% Tween, 100μl streptavidin conjugated to alkaline phosphatase (Boehringer Mannheim GmbH,

22

1:10000 diluted in PBS/0.1% Tween) is added to the wells.
This incubation is followed by washing with PBS/0.1% Tween
and 100mM Tris pH 9.5. p-Nitrophenyl-phosphate (2 mg/ml in
100mM Tris pH 9.5) is used as a substrate for alkaline
phosphatase. The ELISA is read at 405nm.

CD14 is used to compete the peptide-lipid A binding. The
principle of such a binding assay is the same as for the
LALF- or LBP-lipid A competition assays in which a constant
amount of detectable peptide or protein is competed by an
increasing amount of another peptide or protein. Instead of
detecting LALF, bound biotin-labelled peptide is detected
with streptavidin conjugated to alkaline phosphatase, and the
labelled peptide concentration is kept constant while the
unlabelled peptide or protein amount is increased. The
binding of LBP-14 to lipid A binding is strongly competed by
LALF and CD14, while polymyxin B shows only weak competition
activity at the highest concentration used (see Figure 16).
PolP-1 is only slightly above the background determined by
lysozyme as competitor. A very similar result is observed
when LALF-14 is used instead of LBP-14. These results
indicate that the peptides LBP-14 and LALF-14 are able to
compete with CD14 for lipid A binding.

Example 8: Influence of serum on peptide-lipid A binding

To determine the effect of serum proteins on peptide-lipid A
binding, the interaction of labelled peptides is measured in
an ELISA format in the presence of serum concentrations of 1%
and 10% and compared with binding in buffer or medium. For
all peptides tested, LALF-14, LALF-10, LBP-14, LBP-10-1 and
BPI-14, the lipid A binding activity decreases with
increasing serum concentration, but the peptides are still
able to bind lipid A at 10% serum (see Figure 17). As
control, the influence of serum on the LALF-lipid A binding
was investigated, and a serum-dependent decrease of the
LALF-lipid A binding is observed.

Example 9: Influence of serum on the competition of the LALF: lipid A binding by peptide

To investigate whether the specificity of the peptide-lipid A binding is influenced by serum, competition experiments are performed in which LALF-lipid A binding is compared in PBS/0.1% Tween and serum. In all cases only a slight decrease (ca. 5%) of the competition capacity of the peptides in the presence of 10% serum is observed compared to 0% serum (see Figure 18). However the peptides that compete efficiently with LALF for lipid A binding in buffer, such as LALF-14, LBP-14 and polymyxin B, which was used as a control, are also inhibiting in the presence of 10% serum. BPI-14 competes with LALF for lipid A binding to a lesser extent in the presence of 10% serum, and RETL-10 or the polymyxin derived peptide Pol P1 only inhibit slightly above background.

Example 10: Limulus amebocyte Lysate assay

The Limulus amebocyte Lysate test (gelling assay; sensitivity 0.125 EU/ml) and the Chromogenic Limulus amebocyte Lysate test (sensitivity 0.06 EU/ml) are performed according to the manufacturer's instruction (Bio*Whittaker, Walkersville, Maryland) using E.coli 055:B5 lipopolysaccharide. For neutralisation of LPS, peptides (0.1-10 μg/ml) and LPS are incubated for 15 min. at 37°C and then added to the Limulus lysate. In the gelling assay, the positive controls, LALF and polymyxin B, inhibit the assays, the control peptide (control) does not (see Figure 19). L-10, LALF-14, LBP-14, and BPI-14 are also able to inhibit the lysate assay, indicating neutralizing activity (Figure 19). The chromogenic assay allows quantification of the lipopolysaccharide neutralizing capacity of the peptides. LALF and polymyxin B inhibit the reaction up to 95%. Of the peptides, L-10, LALF-14, and LBP14 are best inhibiting (up to 79%). BPI-14 inhibits the chromogenic assay up to 63%, LBP-10-1, a weak

24

competitor of LALF- and LBP-lipid A binding, inhibits the assay up to 50%, while Pol P1, known to inhibit the gelling assay, inhibits the chromogenic assay only up to 25%.

Example 11: Inhibition by peptides of LPS-mediated TNF release by monocytes

Monocytes are purified with a ficoll gradient (d=1.077) from whole blood by centrifugation for 20 min. at 2200 rpm at room temperature. Ficoll is removed by washing 3 times with PBS. Purified monocytes are counted and diluted to 2×10^6 cells/900 μl in medium (RPMI+) or medium with 0.1%-10% human serum. For TNF induction, 2×10^6 monocytes (in 900 μl medium or medium with serum) and 100 μl of LPS (Re-LPS F515; 0.1ng/ml-10ng/ml) or mixtures of LPS with peptides (10 μg/ml) (preincubated for 15 min. at 37°C) are incubated in a 24 well plate (NUNC) for 2 to 5 hr. The supernatant is taken off and spun twice for 2 min. at 1500 rpm, once for 5 min. at 15000 rpm and stored at -20°C for TNF determination. The induced TNF is assayed by ELISA according to the manufacturer's instruction (BioSource International, California) using a 50 μl sample of thawed supernatant.

L-10 and LBP-14 show 55% inhibition of TNF release, under the same conditions as polymyxin B shows 95% inhibition of TNF release (see Figure 20).

Claims

1. An LPS-binding peptide comprising an LPS-binding domain
    comprising at least:

    (a) the amino acid sequence 1-2-3-4-5-6-7-8, wherein the
        numbers represent any of the following amino acids:

        1 = a polar or positively charged amino acid,
            preferably
            C, H, K, N, Q, R, S, T, W, or Y;

        2 = a hydrophobic amino acid, preferably
            A, F, H, I, L, M, V, or W;

        3 = a basic amino acid, preferably H, K, or R;

        4 = a hydrophobic or positively charged amino acid,
            preferably
            A, F, H, I, K, L, M, R, V, or W;

        5 = a hydrophobic, polar, or positively charged amino
            acid, preferably
            A, C, F, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

        6 = a positively charged amino acid, preferably K or
            R;

        7 = A hydrophobic, polar, or positively charged amino
            acid, preferably
            A, C, F, H, I, K, L, M, N, Q, R, S, T, V, W, or Y;

        8 = a hydrophobic or positively charged amino acid,
            preferably
            A, F, H, I, K, L, M, R, V, or W;

    (b) a corresponding inverse amino acid sequence; or

    (c) a variation of said amino acid sequence (a) or (b)
        capable of effectively binding to LPS.

2. The LPS-binding peptide according to claim 1, wherein said
    amino acid sequence (a) is any combination of the amino
    acids seen in the LPS-binding loops of LALF, LBP, or BPI,
    namely:

```
1 = T or R or K
2 = F or W
3 = R or K
4 = R or V or A
5 = L or R or Q
6 = K
7 = W or S or R
8 = K or F
```

3.   The LPS-binding peptide according to claim 1 or 2 with the
     additional feature that the N-terminus is extended by two
     or more amino acids denoted -2 and -1 in which amino acid
     -2, which is the new N-terminus, is taken from the set R,
     K, H, N and Q, and amino acid -1 is any amino acid.

4.   The LPS-binding peptide according to any one of claims 1
     to 3 with the additional feature that the C-terminus is
     extended by addition of cysteine, and the N-terminus is
     extended by two or more amino acids denoted -2 and -1 in
     which amino acid -2, which is the new N-terminus, is taken
     from the set R, K, H, N and Q, and amino acid -1 is
     cysteine, the two cysteines being linked by a disulphide
     bond.

5.   The LPS-binding peptide according to claim 1 or 2, wherein
     said amino acid sequence (a) is the sequence TFRRLKWK.

6.   The LPS-binding peptide according to claim 1 or 2, wherein
     said amino acid sequence (a) is the sequence RWKVRKSFFKLQ.

7.   The LPS-binding peptide according to claim 1 or 2, wherein
     said amino acid sequence (a) is the sequence KWKAQKRFLKMS.

8.   The LPS-binding peptide according to any one of claims 1
     to 7 which is a linear peptide.

9.   The LPS-binding peptide according to any one of claims 1
     to 7 which is constrained to adopt a circular conformation
     by an intramolecular interaction.

10. The LPS-binding peptide according to claim 9 wherein said interaction is a disulfide bond.

11. The LPS-binding peptide according to claim 10 having the amino acid sequence CHYRIKPTFRRLKWKYKGKFWC, CTFRRLKWKC, CRWKVRKSFFKLQC, CRWKVRKSFC, CKWKAQKRFLKMSC, or CKWKAQKRFC wherein the peptide is stabilized by a disulfide bond formed between the terminal cysteine residues.

12. The LPS-binding peptide according to any one of claims 1 to 11 which is detectably labelled.

13. A set of LPS-binding peptides each comprising one or more LPS-binding domains having one of the amino acid sequences according to any one of claims 1 to 12.

14. A DNA sequence encoding a peptide according to any one of claims 1 to 11.

15. A recombinant vector containing a DNA sequence according to claim 14.

16. A microorganism containing a recombinant vector according to claim 15.

17. A method for the production of an LPS-binding peptide according to any one of claims 1 to 12, comprising culturing a microorganism according to claim 16 and recovering said peptide or a fusion protein containing it from the medium.

18. A pharmaceutical composition comprising effective amounts of an LPS-binding peptide according to any of claims 1 to 11 or the set of LPS-binding peptides according to claim 13, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

19. The pharmaceutical composition according to claim 18 for the treatment of Gram-negative sepsis.

20. The pharmaceutical composition according to claim 18 for the treatment of Gram-positive sepsis.

21. The pharmaceutical composition according to claim 18 for the treatment of bacterial infections.

22. The pharmaceutical composition according to claim 18 for the treatment of fungal infections.

23. The pharmaceutical composition according to claim 18 for treatment of heparin-mediated anti-coagulation.

24. The pharmaceutical composition according to claim 18 for the inhibition of angiogenesis.

25. The pharmaceutical composition according to claim 18 for the inhibition of tumour cell proliferation.

26. The pharmaceutical composition according to claim 18 for the inhibition of endothelial cell proliferation.

27. A diagnostic kit containing an LPS-binding peptide according to any one of claims 1 to 12, or a set of LPS-binding peptides according to claim 13.

28. The diagnostic kit according to claim 27 for the determination of LPS or for the diagnosis of septic conditions.

29. The peptides of claims 1 to 12 or the set of LPS-binding peptides according to claim 13, immobilized on a solid support.

30. A method of removing LPS from solution using the immobilized peptides of claim 29.

31. The method according to claim 30 in which said immobilized peptides are used for the removal of bacteria from solution.

Figure 1: schematic drawing of the LALF-loop with the aligned
sequences of LBP and BPI.

Figure 2: Sequence of the peptides comprising different lengths of the LALF-loop.

  (i) amino acids 31 to 52; C-H-Y-R-I-K-P-T-F-R-R-L-K-W-K-Y-K-
     G-K-F-W-C
(ii) amino acids 38 to 45; C-T-F-R-R-L-K-W-K-C

Figure 3: Sequence of the peptides to determine the minimal LPS binding domain in LBP.

  (i) amino acids 90 to 101; C-R-W-K-V-R-K-S-F-F-K-L-Q-C
 (ii) amino acids 90 to 97; C-R-W-K-V-R-K-S-F-C
(iii) amino acids 92 to 99; C-K-V-R-K-S-F-F-K-C

Figure 4: Sequence of the peptides to define the minimal LPS binding domain in BPI.

  (i) amino acids 90 to 101; C-K-W-K-A-Q-K-R-F-L-K-M-S-C
(ii) amino acids 90 to 97; C-K-W-K-A-Q-K-R-F-C

Figure 5: sequence alignment of LALF residues 32 to 50 with the inverse sequence of LBP and BPI from amino acid 103 to 85.

	32																		50
LALF:	H	Y	R	I	K	P	T	F	R	R	L	K	W	K	Y	K	G	K	F

	103																		85
LBP:	S	G	Q	L	K	F	F	S	K	R	V	K	W	R	G	Q	V	R	I

	103																		85
BPI:	N	G	S	M	K	L	F	R	K	Q	A	K	W	K	G	S	I	K	I

Figure 6: Sequence of the peptides in the inverse orientation of the LALF, LBP and BPI sequences.

  (i) LALF amino acids 45 to 38; C-K-W-K-L-R-R-F-T-C
 (ii) LBP amino acids 97 to 90; C-F-S-K-R-V-K-W-R-C
(iii) BPI amino acids 97 to 90; C-F-R-K-Q-A-K-W-K-C

Figure 7: Sequence of the peptides in which point mutations (underlined) are introduced in the LPS-binding peptides derived from LBP.

  (i) mutated LBP amino acids 90 to 97; C-<u>S</u>-<u>F</u>-K-R-V-K-W-<u>K</u>-C
(ii) mutated inverted LBP amino acids 90 to 97; C-<u>K</u>-W-K-V-R-
3   K-<u>F</u>-<u>S</u>-C

FIGURE 8

FIGURE 9

FIGURE 10

FIGURE 11

FIGURE 12

FIGURE 13

FIGURE 14

FIGURE 15

FIGURE 16

FIGURE 17

FIGURE 18

FIGURE 19

PEPTIDE	GELLING ASSAY
ENP	+
L-10	+
LALF-14	+
LBP-14	+
BPI-14	+
POLYMYXIN B	+
CONTROL	-

FIGURE 20

(54) Title: INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE AID OF FOREIGN T-CELL EPITOPES

(57) Abstract

A novel method for utilizing the immune apparatus to remove and/or down-regulate self-proteins consists in inserting one or more foreign T-cell epitopes in such proteins by molecular biological means, thereby rendering said proteins immunogenic. The modulated self-proteins can be used as autovaccines against undesirable proteins in humans or animals, said autovaccine being useful as vaccines against a number of diseases, e.g. cancer, chronic inflammatory diseases, rheumatoid arthritis, inflammatory bowel diseases, allergic symptoms or diabetes mellitus.

Cloning strategy for murine TNFα mutants.

Restriction enzyme symbols: E: EcoRI, B:BamHI, N: NcoI, D: DraIII.

- 1 -

INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE AID OF FOREIGN

T-CELL EPITOPES
_____

5   Background of the invention

       This invention concerns a novel method for utilizing the
       immune apparatus to remove and/or down-regulate self-
       proteins, the presence of which somehow is unwanted in the
10   individual. These could be proteins which are causing
       disease and/or other undesirable symptoms or signs of
       disease. Such proteins are removed by circulating auto-
       antibodies which specifically are induced by vaccination.
       This invention describes a method for developing such
15   autovaccines.

       Introduction

       Physiologically, the vertebrate immune system serves as a
20   defence mechanism against invasion of the body by infec-
       tious objects such as microorganisms. Foreign proteins are
       effectively removed via the reticuloendothelial system by
       highly specific circulating antibodies, and viruses and
       bacteria are attacked by a complex battery of cellular and
25   humoral mechanisms including antibodies, cytotoxic T
       lymphocytes, Natural Killer cells, complement etc. The
       leader of this battle is the T helper (T_H) lymphocyte
       which, in collaboration with the Antigen Presenting Cells
       (APC), regulate the immune defence via a complex network
30   of cytokines.

       Normally the individual's own proteins (the so-called
       self- or autoproteins) are not attacked by the immune
       apparatus. The described events thus generally are
35   beneficial to the individual, but in rare cases the
       process goes wrong, and the immune system turns towards

- 2 -

the individual's own components, eventually leading to an
autoimmune disease.

The presence of some self-proteins is, however, inex-
pedient in situations where they, in elevated levels,
induce disease symptoms. High levels of immunoglobulins of
the IgE class are e.g. known to be important for the
induction of type I allergy, and tumor necrosis factor α
(TNFα) is known to be able to cause cachexia in cancer
patients and patients suffering from other chronic
diseases (H.N. Langstein et al., Cancer Res. 51, 2302-
2306, 1991). TNFα also plays important roles in the
inflammatory process (W.P. Arend et al., Arthritis Rheum.
33, 305-315, 1990). Hormones in sex-hormone dependent
cancer are other examples of proteins which are unwanted
in certain situations. This invention concerns a method
for the development of autovaccines against such proteins.

Others have developed autovaccines by conjugating self-
proteins or appropriate synthetic peptides derived from
these to large, foreign carrier proteins. Talwar et al.
(G.P. Talwar et al, Int. J. Immunopharmacol. 14, 511-514,
1992) have been able to prevent reproduction in women
using a vaccine consisting of a conjugate of human
chorionic gonadotropin and tetanus toxoid. There are also
other examples of such autoimmunogenic conjugates which
have been used therapeutically in man and in animal
models (D.R. Stanworth et al., Lancet 336, 1279-1281
(1990)). In the present invention the production of such
conjugates between the self-proteins and foreign proteins
is not necessary in order to obtain strong autoantibody
responses. This has several advantages.

The technical field

T_H lymphocytes recognize protein antigens presented on the

- 3 -

surface of the APC. They do not recognize, however, native
antigen per se. Instead, they appear to recognize a com-
plex ligand consisting of two components, a "processed"
(fragmented) protein antigen (the so-called T cell epi-
5   tope) and a Major Histocompatibility Complex class II
molecule (O. Werdelin et al., Imm. Rev. 106, 181 (1988)).
This recognition eventually enables the T_H lymphocyte
specifically to help B lymphocytes to produce specific
antibodies towards the intact protein antigen (Werdelin et
10   al., supra). A given T cell only recognizes a certain
antigen-MHC combination and will not recognize the same or
another antigen presented by a gene product of another MHC
allele. This phenomenon is called MHC restriction.

15   Self-proteins are also presented by the APC, but normally
such fragments are ignored or not recognized by the T
helper lymphocytes. This is the reason why individuals
generally do not harbour autoantibodies in their serum.

20   It is, however, possible artificially to induce antibodies
against self-proteins. This can be done, as previously
mentioned, by covalent conjugation of the self-protein to
an appropriate carrier protein as e.g. tetanus toxoid or
key-hole limpet hemocyanin. During the processing of such
25   conjugates in the APC, the necessary T_H lymphocyte
stimulatory epitopes are provided from the foreign protein
eventually leading to the induction of antibodies against
the self-protein as well as against the carrier protein.
One disadvantage of using this principle is, however, that
30   the antibody response towards the self-protein will be
restricted due to shielding of epitopes by the covalently
linked carrier protein. Another disadvantage is the
increased risk of inducing allergic side-effects due to
the contemporary induction of a very strong antibody
35   response against the foreign carrier protein. This strong
antibody response might also be the reason why this method

- 4 -

is not as efficient as observed in the method according to the invention.

Other researchers have coupled a single peptide T cell epitope chemically to a self-protein and managed to induce an autoantibody response with MHC restriction to that particular T cell epitope (S. Sad et al., Immunology 76, 599-603, 1992). This method seems to be more effective compared to coupling of large carrier proteins. However, it will only induce antibodies in a population expressing the appropriate MHC molecules. This means that a rather large number of T cell epitopes has to be coupled to the self-protein which will eventually disturb the B cell epitopes on the surface of the self-protein. Extensive conjugation of proteins may furthermore have the opposite effect with regard to immunogenicity (international patent application No. WO 87/00056) and the surface exposed peptide T cell epitopes may be destroyed by proteolytic enzymes during antigen processing (S. Mouritsen, Scand. J. Immunol. 30, 723, 1989), making that method less efficient than the method of the invention. By this method autoanti-bodies can be induced witin a few weeks (Example 2). Finally, the exact structure of such multi-conjugated self-proteins will not be chemically and pharmaceutically well-defined.

The induction of autoantibodies against TNFα by the method of the present invention has been directly compared to the autoantibody response induced when using a conjugate of TNFα and E. coli proteins, which must contain small single T cell epitope peptides as well as larger foreign carrier proteins. The autoantibody response induced by the method of the invention was induced several weeks earlier and was furthermore of a higher titer (Example 4).

- 5 -

Recently an improved method has been proposed for breaking
the B cell autotolerance by chemical conjugation of B and
optionally also peptide T cell epitopes to a high mole-
cular weight dextran molecule (international patent appli-
5   cation No. WO 93/23076). The disadvantages mentioned
above, however, also hold true for said method, which any-
way is clearly different from the method of the present
invention.

10   Although it has been proposed previously that a well known
strong T cell epitope could be inserted into a foreign
protein using recombinant DNA technology (EP-A2-0 343 460)
or synthetically into a peptide (WO 90/15627) in order to
increase an antibody response towards that protein or pep-
15   tide, it has not been proposed that this could be done
with the purpose of breaking the autotolerance of the
immune system. Using these methods for induction of auto-
antibodies one a priori would expect the same rules to be
true with regard to the above-mentioned limitations of the
20   MHC restriction of the reponse. Surprisingly, however, by
using the method of the invention, it is possible to
induce and equally fast and even a stronger autoantibody
response against TNFα despite the fact that the inserted T
cell epitope used was not restricted to the MHC molecules
25   of the immunized mice (Example 3). The reason for this
observation is not clear but may be due to the appearance
of new MHC binding segments in the mutagenized area in the
self-protein. However, the experiment shown in example 6
demonstrates that this is probably not the case, since
30   synthetic peptides representing overlapping regions of the
implanted ovalbumin T cell epitope in ubiquitin did not
bind strongly to any of the MHC class II molecules of the
H-2^k mice in which this recombinant molecule was highly
immunogenic (Example 5).

35

- 6 -

Most of the potential MHC class II binding segments of a protein are normally cryptic and will not be presented to the host T cells by the antigen presenting cells (S. Mouritsen et al, Scand. J. Immunol. 34, 421, 1991). The observed lacking correspondence between the MHC restriction of the inserted T cell epitope and the restriction of the antibody response could perhaps be due to a general disturbance of the intra-molecular competition of binding to MHC molecules by different self-protein segments. Using the herein described method non-tolerized self-protein segments may be presented to the T cells leading to breaking of the T cell as well as the B cell autotolerance towards the protein. In all the examples described below, a fragment of the self-protein was substituted with a foreign T cell epitope. This deletion followed by a sub-stitution with an other protein fragment minimally obscure the tertiary structure of the self-proteins, but may also contribute strongly to the disturbance of said intra-molecular competition of the MHC class II binding self-segments. This concept is therefore clearly different from the above-mentioned mechanisms and methods. Independently of the operating mechanism of action by the herein described method, it is more technically advantageous com-pared to the known methods for breaking the B cell auto-tolerance, since it is possible to induce antibodies in a broad population of MHC molecules by insertion of a mini-mal number of different foreign T cell epitopes.

The present invention thus concerns the surprising fact that injection of recombinant proteins, which have been appropriately modulated by the insertion of one or more foreign T cell epitopes, induces a profound autoantibody response against said proteins. Surprisingly the antibody response induced is not necessarily restricted to the inserted T cell epitope. By inducing minimal tertiary structural changes in the highly conserved self-protein

- 7 -

ubiquitin, as well as in TNFα, foreign T cell epitopes
having a length of 12-15 amino acids were inserted using
genetic engineering methods. These recombinant proteins
were purified, emulsified in adjuvant and injected into
5   mice. Within only one week an autoantibody response
against ubiquitin could be detected in serum from these
mice. Non-modified, recombinant ubiquitin treated and
injected in the same way was not able to induce a
response.
10

By using this principle for developing vaccines against
undesirable proteins, the risk of inducing allergic side-
effect is reduced, and toxic self-proteins such as TNFα
can simultaneously be detoxified by removing or mutating
15   biologically active protein segments. The epitope-shield-
ing effect described above is not a problem, and autoanti-
bodies against ubiquitin were induced much faster as com-
pared to the known technique, in which the self-protein is
conjugated to a carrier protein or peptide. Importantly,
20   by this method it furthermore seems possible to tempora-
rily break the autotolerance of the T cells as well as
that of the B cells of the individual, and such recom-
binant proteins will be self-immunogenic in a large popu-
lation expressing many different MHC class II molecules.
25

The vaccine of the invention consists of one or more self-
proteins modulated as described above and formulated with
suitable adjuvants, such as calcium phosphate, saponin,
quil A or biodegradable polymers. The modulated self-
30   proteins may be prepared as fusion proteins with suitable,
immunologically active cytokines, such as GM-CSF or
interleukin 2.

The autovaccine may i.a. be a vaccine against TNFα or γ-
35   interferon for the treatment of patients with cachexia,
e.g. cancer patients, or a vaccine against IgE for the

- 8 -

treatment of patients with allergy. Furthermore, it may be
a vaccine against TNFα, TNFβ or interleukin 1 for the
treatment of patients with chronic inflammatory diseases.

5   The invention is illustrated in the following examples.

Example 1. Cloning of foreign T cell epitopes into a gene
coding for ubiquitin.

10

An overview of this procedure is shown in fig. 1 using the
T cell epitope MP7 as example. The gene sequences
representing MP7 (MP7.1-C and MP7.1-NC) were synthesized
as two complementary oligonucleotides designed with
15   appropriate restriction enzyme cloning sites. The amino
acid sequence of MP7 is PELFEALQKLFKHAY. The oligonucleo-
tides were synthesized using conventional, automatic solid
phase oligonucleotide synthesis and purified using agarose
gel electrophoresis and low melting agarose. The desired
20   bands were cut out from the gels, and known quantities of
oligonucleotides were mixed, heated to 5°C below their
theoretical melting point (usually to approximately 65°C)
for 1-2 hours, and slowly cooled to 37°C. At this
temperature the hybridized oligonucleotides were ligated
25   to the vector fragments containing part of the ubiquitin
gene. The subsequent analysis of positive clones using
restriction fragment analysis and DNA sequencing was done
by conventional methods ("Molecular Cloning", Eds.: T.
Maniatis et al. 2 ed. CSH Laboratory Press, 1989).

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Example 2. Induction of autoantibodies against ubiquitin
by vaccination with modified ubiquitin molecules.
----

35   Genes containing the foreign T cell epitopes were
expressed in E. coli strain, AR58 under control by the

- 9 -

heat sensitive λ repressor regulated promotor. Expression
of the recombinant ubiquitin proteins were verified using
a polyclonal anti-ubiquitin antibody and Western-blotting
("Antibodies", Eds.: D. Harlow et al., CSH Laboratory
5   Press, 1988). The recombinant proteins were purified using
conventional methods (Maniatis et al., supra).

Mice were injected i.p. with 100 μg of ubiquitin or its
analogs in PBS emulsified in Freunds Complete adjuvant.
10   Booster injections of the same amount of antigen
emulsified 1:1 in Freunds Incomplete adjuvant were
performed i.p. at days 14 and 28. Five Balb/c mice in each
group were examined and blood samples were examined for
the presence of anti-ubiquitin antibodies on day 7, 14,
15   21, 28, 35, and 42 using conventional ELISA methodology.

The results exemplified by the antibody response against
two different ubiquitin molecules containing the T cell
epitopes OVA(325-336) and HEL(50-61), respectively, are
20   shown in fig. 2. The amino acid sequence of the inserted
OVA(325-336) epitope is: QAVHAAHAEINE and the amino acid
sequence of the HEL(50-61) epitope is STDYGILQINSR.

A clear antibody response against native ubiquitin could
25   be detected within only one week from the first injection
of antigen reaching a maximum within 2 weeks. Anti-
ubiquitin antibodies produced in rabbits by covalently
conjugating ubiquitin to bovine immunoglobulin reached
maximum values after a much longer immunization period
30   (data not shown).

The antibody response against self-proteins can be
increased even more by injecting self-proteins containing
foreign T cell epitopes, as described in example 1, as
35   fusion proteins with immunologically active cytokines such
as e.g. granulocyte and monocyte colony stimulating factor

- 10 -

(GM-CSF) or interleukin 2.

Example 3. Induction of autoantibodies against tumor
necrosis factors (TNF) by vaccination with appropriately
5   modified TNF molecules.

       The gene coding for the structural part of the murine TNFα
       protein (MR101) was obtained by Polymerase Chain Reaction
10   (PCR) cloning of the DNA. In the MR103 TNFα mutant the
       ovalbumin (OVA) H-2^d restricted T cell epitope sequence
       325-334 (QAVHAAHAET) replaces the amino acids 26-35 in the
       cloned TNFα sequence, a substitution of an amphiphatic α-
       helix. Substitutions in this region of the TNFα detoxifies
15   the recombinant protein (X. Van Ostade et al., Nature 361,
       266-269, 1993). In the MR105 TNFα mutant the H-2^k re-
       stricted T cell epitope from hen eggwhite lysozyme (HEL),
       amino acid sequence 81-96 (SALLSSDITASVNCAK) replaces the
       amino acids 5-20 in the cloned TNFα sequence. In the MR106
20   TNFα mutant the same epitope, amino acid sequence 81-95
       (SALLSSDITASVNCA) replaces the amino acids 126-140 in the
       cloned TNFα sequence. The genetic constructions are
       described in Fig. 3. Different techniques compared to the
       technique described in example 1 were used for exchanging
25   parts of the TNFα gene with DNA coding for T cell epi-
       topes. The MR105 and 106 constructs were made by intro-
       ducing the mutant sequence by PCR recloning a part of the
       TNFα gene flanking the intended site for introducing the T
       cell epitope. The mutant oligonucleotide primer contained
30   both a DNA sequence homologous to the TNFα DNA sequence
       as well as a DNA sequence encoding the T cell epitope. The
       PCR recloned part of the TNFα gene was subsequently cut
       with appropriate restriction enzymes and cloned into the
       "wild type" MR101 gene. the MR103 construction was made by
35   a modification of the "splicing by overlap extension" PCR
       technique (R. M. Horton et al., Gene 77, 61, 1989). Here

- 11 -

two PCR products are produced, each covering a part of the TNFα gene, and additionally each PCR product contains half of the T cell epitope sequence. The complete mutant TNFα gene was subsequently made by combining the two PCR products in a second PCR. Finally, the complete genetic constructions were inserted into protein expression vectors. Subsequently, all genetic constructions were analyzed by restriction fragment analysis and DNA sequencing using conventional methods ("Molecular Cloning", Eds,: T. Maniatis et al. 2.ed. CSH Laboratory Press, 1989). The recombinant proteins were expressed in E.coli and purified by conventional protein purification methods.

Groups of BALB/c (MHC haplotype, H-2^d) and C3H (MHC haplotype, H-2^k) mice, respectively, were immunized subcutaneously with 100 μg of semi-purified MR103 and MR106 emulsified in Freunds' complete adjuvant. Every second week the immunizations were repeated using incomplete Freunds' adjuvant. All mice developed an early and strong antibody response against biologically active MR101. This was measured by a direct ELISA method using passively adsorbed 100% pure MR101 (Fig. 4). Control mice immunized with MR101 and PBS, respectively, showed no antibody reactivity towards MR101.

Strinkingly, the response was not MHC restricted corresponding to the implanted T cell epitopes, since both mice strains responded well to MR103 and MR106 (Fig. 4). Taken together these results illustrate (a) the ability of the method of the invention to induce autoantibodies towards a secreted autoprotein and (b) the improved efficiency of the herein described method with regard to inducing a response in a broader MHC population than predicted by the MHC binding ability of the inserted T cell epitopes. The immune response against the recombinant proteins MR103 and MR106 was much stronger and more high-titered compared to

- 12 -

aldehyde conjugated MR101 (see Example 4).

Example 4. Induction of autoantibodies against TNFα by the
method of the invention compared to conjugation to E. coli
5   proteins.

Semi-purified recombinant murine TNFα (MR101) was conju-
gated to E. coli proteins in PBS, pH 7.4, using 0.5%
10   formaldehyde. Conjugation of the proteins was confirmed by
SDS-PAGE. These conjugates were subsequently used for
immunization of C3H mice. Another group of C3H mice was
vaccinated only with semi-purified non-conjugated MR105,
and about 100 μg of recombinant TNFα were emulsified 1:1
15   in Freunds' complete adjuvant and injected subcutaneously
in each mouse. MR105 is biologically inactive as judged by
the L929 bioassay for TNFα. In subsequent immunizations
every second week incomplete Freunds' adjuvant was used.
Both groups eventually developed autoantibodies against
20   highly purified biologically active MR101 as determined by
ELISA, but the immune response against non-conjugated
MR105 was induced earlier and was of a higher titer (Fig.
5).

25   Example 5. The possible MHC class II binding of peptides
representing overlapping sequences of self-protein as well
as of the ovalbumin T cell epitope inserted in ubiquitin.
----

30   Peptide-MHC complexes were obtained by incubaing ^{125}I-
labelled peptide (10-100 nM) with affinity purified MHC
class II molecules (2-10 μM) at room temperature for 3
days (S. Mouritsen, J. Immunol. 148, 1438-1444, 1992). The
following peptides were used as radiolabelled markers of
35   binding: Hb(64-76)Y which binds strongly to the E^k
molecule and HEL(46-61)Y which binds strongly to the A^k

- 13 -

molecule. These complexes were co-incubated with large
amounts of cold peptide (> 550 μm) which is sufficient to
inhibit totally all immunologically relevant MHC class II
binding. Either the same peptides were used, or three
5   different overlapping peptides were used, said peptides
representing the flanking regions as well as the entire
OVA(325-336) T cell epitope which was substituted into
ubiquitin (see Example 2). The three peptides were:
TITLEVEPSQAVHAA (U(12-26)), PSQAVHAAHAEINEKE (U(19-34))
10   and HAEINEKEGIPPDQQ (U(27-41)). The reaction buffer
contained 8 mM citrate, 17 mM phosphate, and 0.05% NP-40
(pH 5) and peptide-MHC class II complexes were separated
(in duplicate) from free peptide by gel filtration using
G25 spun columns. Both the radioactivities of the excluded
15   "void" volume and of the included volume were measured by
gamme spectrometry. The competitive inhibition of maximal
binding (in percent) by addition of cold peptide was
calculated. The results are shown in Table I.

20   Table I.

Peptid/ MHC	Hb(64-76)	HEL(46-61)	U(12-26)	U(19-34)	U(27-41
A^k	28.6	<u>97.4</u>	35.3	44.6	7.8
E^k	<u>92.6</u>	0.0	45.6	12.2	0.0

30

It can be seen that total inhibition of the binding of the
radiolabelled peptides Hb(64-76)Y and HEL(46-61)Y to E^k
and A^k respectively could only be achieved using cold
versions of the same peptides. Although some inhibition of
35   binding was seen by U(12-26) and U(19-434) using these
extreme amounts of cold peptide, it is likely that the

- 14 -

affinity of these peptides to the H-2^k MHC class molecules
is very low. Therefore this seems not to be sufficient to
explain the strong immunogenicity in the H-2^k mouse strain
of the ubiquitin analog containing the ovalbumin T cell
5   epitope. More likely, other and previously non-tolerized
self-epitopes are presented to the T cell in these ani-
mals.

Example 6. Treatment of diabetes of inflammatory disease
10 by vaccination with appropriately modified TNFα molecules.

Genes coding for TNFα are modified by insertion of appro-
priate gene segments coding for T cell epitopes derived
15 from e.g. tetanus toxin or influenza hemagglutinin. Such
genes are expressed in appropriate expression vectors in
e.g. E. coli or insect cells. The recombinant TNFα pro-
teins were purified using conventional methods ("Molecular
Cloning", Eds.: T. Maniatis et al. 2. ed. CSH Laboratory
20 Press, 1989).

Optionally such recombinant proteins can be coupled to
immunologically active cytokines such a GMCSF or
interleukin 2.
25

The recombinant proteins can be formulated with appro-
priate adjuvants and administered as an anti-TNFα vaccine
to patients suffering from diseases where TNFα is
important for the pathogenesis. The induced anti-TNFα
30 antibodies will thereby affect the diseases.

One example of said diseases is the chronic inflammatory
diseases such as e.g. rheumatoid arthritis where TNFα is
believed to play an important role (reviewed in: F.M.
35 Brennan et al., Br. J. Rheumatol. 31, 293-298, 1992). TNFα
is also believed to play an important role in the cachec-

- 15 -

tic conditions seen in cancer and in chronic infectious
diseases such as AIDS (reviewed in M. Odeh. J. Intern.
Med. 228, 549-556, 1990). It is also known that TNF
participates in septic shock (reviewed in: B.P. Giroir,
Crit. Care. Med., 21, 780-789, 1993). Furthermore, it has
been shown that TNFα may paly a pathogenetic role in the
development of type II diabetes mellitus (CH Lang et al.,
Endocrinology 130, 43-52, 1992).

Legends to figures

Fig. 1. Schematic overview of the cloning strategy used
in the construction of a ubiquitin gene with an implanted
foreign T cell epitope (MP7). Restriction enzyme
digestions, hybridization and ligation procedures are
indicated with arrows. Fragment sizes are shown in
parentheses.

Fig. 2. Reactivity toward immobilized bovine ubiquitin in
sera from mice immunized with recombinant ubiquitin and
analogs containing the implanted T cell epitopes OVA(323-
339) and HEL (50-61), respectively. Fig. 2a) sera from
Balb/c mice immunized with recombinant ubiquitin
containing OVA(325-336). Fig. 2b) sera from Balb/c mice
immunized with recombinant ubiquitin containing the T cell
epitope HEL(50-61). Fig. 2c) sera from Balb/c mice
immunized with recombinant non-modified ubiquitin. Sera
(diluted 1:100) were tested in a standard ELISA assay
using non-modified bovine ubiquitin immobilized on the
solid phase.

Fig. 3. Schematic overview of the cloning strategy used in
the construction of the recombinant TNFα mutants. PCR
products and restriction enzyme digestions are indicated.

- 16 -

Fig. 4. Induction of TNFα autoantibodies by vaccination of
Balb/c or C3H mice with semipurified MR103 and MR106. The
antibody titers were measured by ELISA and expressed as
arbitrary units (AU) referring to a strong standard anti-
5   serum from one mouse. The plotted values represent a mean
titer for 5 animals. Freunds complete adjuvant was used as
adjuvant for the first immunization. All subsequent immu-
nizations at 14 days internvals were done with Freunds
incomplete adjuvant. Mice immunized in parrallel with
10   native MR101 in PBS did not develop detectable TNFα auto-
antibodies (data not shown). Non-detectable antibody
titers were assigned the titer value 1.

Fig. 5. Anti TNFα autoantibodies induced by vaccination
15   with non-conjugated MR105 and MR101 conjugated to E. coli
proteins, respectively. C3H mice and Balb/c mice were
immunized with both preparations. The immunizations,
measurements and calculations of mean antibody titers were
done as described in example 4.

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- 17 -

C l a i m s :
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1.   A method for the modulation of self-proteins by
inducing antibody responses against such proteins,
c h a r a c t e r i z e d in that one or more foreign T
cell epitopes are inserted in such proteins by molecular
biological means, thereby rendering said proteins
immunogenic.

2.   A method according to claim 1, c h a r a c t e r -
i z e d in that immunodominant T cell epitopes from
tetanus toxoid or diphtheria toxoid are inserted in said
proteins.

3.   An autovaccine against undesirable proteins in humans
or animals, c h a r a c t e r i z e d in that it consists
of one or more self-proteins modulated according to claim
1 or 2 and formulated with pharmaceutically acceptable
adjuvants, such as calcium phosphate, saponin, quil A and
biodegradable polymers.

4.   An autovaccine according to claim 3, c h a r a c -
t e r i z e d in that the modulated self-proteins are
prepared as fusion proteins with suitable, immunologically
active cytokines, such as GM-CSF or interleukin 2.

5.   An autovaccine according to claim 3, c h a r a c -
t e r i z e d in that it is a vaccine against TNFα or γ-
interferon for the treatment of patients with cachexia,
e.g. cancer patients.

6.   An autovaccine according to claim 3, c h a r a c -
t e r i z e d in that it is a vaccine against IgE for the
treatment of patients with allergy.

- 18 -

7.   An autovaccine according to claim 3, c h a r a c -
t e r i z e d in that it is a vaccine against TNFα, TNFβ
or interleukin 1 for the treatment of patients with
chronic inflammatory diseases.

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8.   An autovaccine according to claim 7, c h a r a c -
t e r i z e d in that it is a vaccine for treatment of
patients with rheumatoid arthritis or inflammatory bowel
disease.

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9.   An autovaccine according to claim 3 or 4, c h a -
r a c t e r i z e d in that it is a vaccine against TNFα
for the treatment of diabetes mellitus.

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FIG. 1

FIG. 2

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Cloning strategy for murine TNFα mutants.

Restriction enzyme symbols: E: EcoRI, B:BamHI, N: NcoI, D: DraIII.

FIG. 3

SUBSTITUTE SHEET

Anti TNFα auto-antibodies

FIG. 4

Anti-TNFα autoantibodies

FIG. 5

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 39/00, A61K 39/385, C07K 19/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, US FULLPAT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	National Library of Medicine database, Medline, File Med 93, NLM Accession no. 93013892, Sad S. et al: "Bypass of carrier-induced epitope--specific suppression using a T-helper epitope", & Immunology 1992 Aug; 76(4): 599-603	1-3
Y		1-9
	--	
X	WO, A1, 9219746 (CSL LIMITED), 12 November 1992 (12.11.92), page 3, line 3 - line 14, see claims	1-3
Y		1-9
	--	

| X | Further documents are listed in the continuation of Box C. | X | See patent family annex. |

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 1995	01 -02- 1995

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Carl Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

	International application No.
	PCT/DK 94/00318

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	Methods in Enzymology, Volume 178, 1989, M. J. Francis et al, "Peptide Vaccines Based on Enhanced Immunogenicity of Peptide Epitopes Presented with T-Cell Determinants or Hepatitis B Core Protein" page 659 - page 676	1-3
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INTERNATIONAL SEARCH REPORT

	International application No.
	PCT/DK 94/00318

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO, A1, 8912458 (CELL MED, INC.), 28 December 1989 (28.12.89), see pages 1-3, page 8, last two lines, page 12, page 28, third paragraph and claims 1,4,9, 22 and 29	1,3,4,7-9
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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00318

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0269455 (TAKEDA CHEMICAL INDUSTRIES, LTD.), 1 June 1988 (01.06.88), see page 5, lines 6-28 and claims	6
Y	US, A, 4684623 (JAMES W. LARRICK ET AL), 4 August 1987 (04.08.87)	5,7-9
Y	WO, A2, 8807869 (STICHTING REGA VZW), 20 October 1988 (20.10.88)	5
A	WO, A1, 9102005 (TURANO, ADOLFO), 21 February 1991 (21.02.91)	5
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Y	WO, A1, 9101330 (SCHERING CORPORATION), 7 February 1991 (07.02.91)	4
A	US, A, 4772685 (JOHN A. SCHMIDT ET AL), 20 Sept 1988 (20.09.88)	1,3,4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☒ The additional search fees were accompanied by the applicant's protest.

           ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

1)   Claims 1-3
      A method for the modulation of self proteins by insertion
      of a T-cell epitope into the protein and an autovaccine.

2)   Claims 4,5,7 and 8
      An autovaccine comprising a fusion protein of a foreign
      T-cell epitope and a self protein prepared as fusion
      proteins with suitable, immunologically active cytokines.

3)   Claim 6
      Autovaccine comprising a foreign T-cell epitope inserted
      in IgE

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9219746	12/11/92	AU-B-	634379	18/02/93
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(54) Title: AMELIORATION OF AMNESIA IN ALZHEIMER'S DISEASE CAUSED BY DEPOSITION OF AMYLOID β PROTEIN

(57) Abstract

Three non-amnestic and non-memory enhancing peptides, Asp Phe Phe Val Gly (SEQ ID NO: 1), Gln Phe Val Gly (SEQ ID NO: 2), and Ala Ile Phe Thr (SEQ ID NO: 3), that block the amnestic effects of β-(12-28), a peptide homologous to amyloid β protein (Aβ) are disclosed. This invention relates to amelioration of amnesia and other neurotoxicity in Alzheimer disease (AD) caused by deposition of Aβ and, therefore, relates to attenuation of the disease process and consequential improvement of the quality of life for individuals suffering from AD.

-1-

AMELIORATION OF AMNESIA IN ALZHEIMER'S DISEASE CAUSED BY DEPOSITION OF AMYLOID β PROTEIN

This application is a continuation of United States application Serial No. 08/127,904 filed 29 September 1993.

FIELD OF THE INVENTION

This invention relates to amelioration of amnesia in Alzheimer disease (AD) caused by deposition of amyloid ß protein (Aß) and, therefore, to attenuation of the disease process and consequential improvement of the quality of life for individuals suffering from AD. More particularly the invention relates to prevention of deterioration of memory and quality of life in AD patients by administration of the peptides Asp Phe Phe Val Gly (SEQ ID NO: 1), Gln Phe Val Gly (SEQ ID NO: 2), and Ala Ile Phe Thr (SEQ ID NO: 3) or amides or esters thereof. Administration of these substances to human individuals with AD can enhance memory and attenuate progression of the disease, in this way improving the quality of life.

DEFINITIONS

The following abbreviations are used:

Aß = = amyloid ß protein
FAAT = footshock active avoidance training
ICV = intracerebroventricular
Ala = alanine
Cys = cysteine
Asp = aspartic acid
Glu = glutamic acid
Phe = phenylalanine
Gly = glycine
His = histidine
Ile = isoleucine

-2-

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Lys  = lysine
Leu  = leucine
Met  = methionine
Asn  = asparagine
Pro  = proline
Gln  = glutamine
Arg  = arginine
Ser  = serine
Thr  = threonine
Val  = valine
Trp  = tryptophan
Tyr  = tyrosine
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BACKGROUND OF THE INVENTION

Much data suggests that in Alzheimer disease (AD) there may be genetically and/or environmentally induced defects in the enzymatic machinery involved in degradation of amyloid precursor protein (APP) (for reviews, see refs. 1 and 2). Alternative splicing of mRNAs gives rise to at least five forms of APP, two of which possess a Kunitz-type protease inhibitory domain. Normal lysosomal processing of APPs involves highly coordinated sequences of desulfation, dephosphorylation, deglycosylation, and proteolytic splitting. The APPs may belong to a family of polypeptide precursors or polyproteins that upon processing give rise to a number of different bioactive peptides that may act individually or in concert to regulate cellular activation (3-5). The processing of the parent molecules and/or the extracellular secretion of the resulting subunits may vary with species, tissue, age, hormonal status, extent of phosphorylation (6), etc. Although the APPs may be cell-surface receptors (7, 8), some of the peptidic fragments derived from them may be ligands (9) for specific membrane sites.

-3-

To some extent in normal aging and to greater extent in AD and in adult Down syndrome, abnormal processing of APP gives rise to an insoluble self-aggregating 42-amino acid polypeptide designated as amyloid ß protein (Aß) that is found in amyloid (10-14). The extent of Aß deposition correlates with the degree of neuronal damage, cognitive impairment, and memory loss (15-18). Amyloid-like fibrils arise readily in vitro under physiological conditions even from the following smaller peptides homologous to Aß: ß-(1-28) (N-terminus residues 1 to 28), [Gln^{11}]ß-(1-28), ß-(12-18), and ß-(18-28) (19-21). Extensive stacks of ß-pleated sheets are formed from the latter peptide (21). Functional deficits arise in AD from damage to nerve circuitry per se, which is known to occur in late phases of the disease (22, 23). It also is possible that binding of Aß and related peptides to components of the extracellular matrix (e.g., proteoglycans (24)) or to receptors on endothelial, glial, or neuronal cells in particular brain regions could have disruptive effects on neuronal communications at earlier stages of the disease when the deposits of these substances are diffused and typical cytopathological evidence of AD often is absent.

It has been demonstrated (25) that Aß and, perhaps, smaller peptidic fragments thereof that are responsible for binding of Aß to cell membranes or components of the extracellular matrix may have amnestic effects upon appropriate administration to experimental animals. Hence, soluble peptides or structurally mimetic nonpeptidic substances can be devised to antagonize the binding of the Aß and thus alleviate some of the symptoms of AD not caused b actual physical destruction of neural circuitry. Progression may also be attenuated by such substances.

-4-

SUMMARY OF THE INVENTION

This invention involves the discovery that three peptides, Asp Phe Phe Val Gly (SEQ ID NO: 1), Gln Phe Val Gly (SEQ ID NO: 2), and Ala Ile Phe Thr (SEQ ID NO: 3), overcome the amnestic effects of ß-(12-28), a peptide homologous to Aß that is as potently amnestic as Aß (25) and which shows amyloid-like aggregation similarly to Aß (19-21). No other substances are known which serve this purpose.

DETAILED DESCRIPTION OF THE INVENTION

Screening of various peptides which neither are significantly amnestic nor memory-enhancing in memory-testing paradigms in mice resulted in the discovery of three peptides that blocked the amnestic effects of ß-(12-28), a peptide homologous to Aß. Administration of the peptides (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3) or their esters or amides orally, subcutaneously, intravenously, transcutaneously, intrathecally, sublingually, rectally, or intracisternally leads to an amelioration of symptoms in Alzheimer disease by decreasing deposition of amyloid in the brain.

This discovery facilitates the development of substances that can antagonize binding of Aß to neural structure and thus attenuate symptoms and progression of Alzheimer disease. Similarity in brain function in various mammals, including human beings, and previous neurological experience, indicates that the three peptides discovered to block the amnestic effects of ß-(12-28) and derivatives and variants including esters and amides thereof will be effective therapeutic substances in human beings with Alzheimer disease. In no known instance have such substances been proposed for this purpose.

-5-

DESCRIPTION OF THE FIGURES

Figure 1 depicts an antagonism by Asp Phe Phe Val Gly (SEQ ID NO: 1) of amnestic effect of ß-(12-28) when administered before or after ß-(12-28) to groups of 15 mice each. SAL = physiological saline (sterile).

EXEMPLIFICATION OF THE INVENTION
Materials and Methods

Test Animals. After one week in the laboratory, CD-1 male mice obtained from Charles River Breeding Laboratories were caged individually 24-28 hours prior to training and remained singly housed until retention was tested one week later. Animal rooms were on a 12-hour light/dark cycle with lights going on at the hour of 0600. Median body weight was 35 g, with a range of 33-38 g. Mice were assigned randomly to groups of ten in the experiments reported in Table 1, groups of 14 in the experiments reported in Table 2, and groups of 15 in Figure 1 and were trained and tested between the hours of 0700 and 1500.

Peptides Tested. The peptides used in these studies were synthesized and analyzed to establish purity by standard methods at the Beckman Research Institute.

Peptides were dissolved in 8% vol/vol dimethyl sulfoxide and diluted to a final concentration of 0.001% dimethyl sulfoxide in saline. Upon testing for retention of FAAT after receiving post-training ICV administration of 2 μl of the above vehicle the mean numbers of trials to criterion ± standard error of the mean (SEM for well trained mice and weakly trained mice were 6.85±0.20 and 9.07±0.25, respectively (see the paragraph below for definition of the two training paradigms).

The experiments below tested whether or not there were amnestic or memory-enhancing effects at 6 nmol of peptide per mouse.

Apparatus, training and Testing Procedures. The
T-maze used for footshock active avoidance training
(FAAT) consisted of a black plastic alley (46 cm
long) with a start box at one end and two goal boxes
(17.5 cm long) at the other. The start box was
separated from the alley be a plastic guillotine door
that prevented movement down the alley until training
began. The alley was 12.5 cm deep and 9.8 cm wide.
An electrifiable stainless steel rod floor ran
throughout the maze.

Mice were not permitted to explore the maze
before training. A block of training trials began
when a mouse was placed in the start box. The
guillotine door was raised and a muffled
doorbell-type buzzer sounded simultaneously;
footshock was 5 seconds later through a scrambled
grid floor shocker (Colbourn Instruments, Model
E13-08). The goal box first entered during the first
set of trials was designated as "incorrect", and
footshock was continued until the mouse entered the
other goal box, which in all subsequent trials was
designated "correct" for the particular mouse. At
the end of each group of trials, the mouse was
removed to its home cage.

As training proceeded, a mouse made one of two
types of responses. A response latency longer than 5
seconds was classed as an escape from the footshock.
A response latency less than or equal to 5 seconds
was considered an avoidance, since the mouse avoided
receiving a footshock. Two exclusion criteria were
applied to reduce learning variability among mice, as
follows. On the first training trials, mice with
escape latencies greater than 20 seconds were
discarded. Mice not having at least one errorless
escape latency between 1.5 and 3.5 seconds on
training trials 3 or 4 were excluded. The total

-7-

exclusions were fewer than 15%. Mice received five
such training trials. One week after training and
post-trial administration of vehicle alone or vehicle
containing test substance, T-maze training was
resumed until each mouse made five avoidance
responses in six consecutive training trials (trials
to criterion). The recall score was taken to be the
percentage of tested mice remembering original
training.

Well-trained animals (recall score approximately
80%) were used to determine whether or not
administered substances could cause amnesia. In
these instances, training was performed under
conditions that tend to maximize learning (sound
intensity, 65 decibels; footshock current, 0.35 mA;
intertrial interval, 45 seconds). In the cases in
which it was desired to detect whether or not there
was an enhancing effect on memory, training
conditions were adjusted so that the initial recall
score in vehicle controls was only approximately 20%
(sound intensity, 55 decibels; footshock current,
0.30 mA; intertrial interval, 30 seconds).

Surgical Procedure in Preparation for
Intracerebroventricular (ICV) Administration of
Substances. ICV injection was the mode of
administration of test substances because this
eliminates problems of differential penetration of
the blood-brain barrier. The following procedure was
performed 24-48 hours prior to training. A single
hole was drilled through the skull over the third
ventricle (-0.5 mm relative to bregma, 0.5 mm right
of central suture) while the mouse, appropriately
anesthetized with methoxyflurane, was held in a
stereotaxic instrument. The third ventricle was
chosen as site of ICV drug injection because only a
single injection is required and the drug quickly

-8-

reaches limbic system structures, believed to be
associated with memorial processes. Immediately
after training, mice were anesthetized with
enflurane, a short acting anesthetic, and given an
ICV injection of 2 μl of vehicle alone or test
substance in vehicle delivered over a 30-second
period through a 31-gauge needle attached to a 10-μl
syringe; the injection was given within 2-3 minutes
after the training. Accuracy of injection was
determined to be greater than 95% by due injection,
monitored regularly.

Statistical Treatment of Data. All of the
results are expressed in terms of the mean and
standard errors of the mean (SEM). Significance of
overall effects of treatment was determined by
one-way analysis of variance (ANOVA) run on trials to
criterion. Dunnett's t-test was used to make
multiple comparison of individual test groups with
control groups. See Bruning, J.E., et al., in
Computational Handbook of Statistics, 2d ed., Scott,
Foreman and Co., Glenview, pp. 18-30, 122-124,
128-130 (1977). Statistical comparison among
experimental groups were made by Bukey's t-test. See
Winer, B.J., Statistical Principles in
Experimentation Design, 2d ed., McGraw-Hill, New
York, pp. 196-210, 397-402 (1971).

RESULTS

Three non-amnestic peptides block the amnestic
effects of ß-(12-28), a peptide homologous to ß/A4).
The following peptides tested under standard
conditions in groups of 15 mice. Each were found to
have no significant amnestic effect in the standard
test with well-trained mice: Phe Phe (SEQ ID NO: 4),
Val Val (SEQ ID NO: 5), Ala Val Phe (SEQ ID NO: 6),
Phe Val Phe (SEQ ID NO: 7), Ala Phe Ile Gly (SEQ ID
NO: 8), Ala Ile Phe Thr (SEQ ID NO: 3), Gly Phe Met

Thr (SEQ ID NO: 9), Asn Leu Ile Thr (SEQ ID NO: 10),
Gln Phe Val Gly (SEQ ID NO: 2), Ser Phe Phe Gly (SEQ
ID NO: 11), Ser Phe Val Gly (SEQ ID NO: 12), Asp Phe
Phe Val (SEQ ID NO: 13), Asp Phe Phe Val Gly (SEQ ID
NO: 1), Lys Leu Val Phe Phe Ala Glu (SEQ ID NO: 14),
and Lys Leu Val Phe Phe (SEQ ID NO: 15). Three of
the above, SEQ ID NOS: 1, 2 and 3, blocked the
amnestic effect of ß-(12-28) (26) on retention of
FAAT when co-administered to groups of ten mice, each
with isomolar amounts (6 nmol) of ß-(12-28)
(Table 1), giving the following values for trials to
criterion ±SEM and p values for comparison with
ß-(12-28): ß-(12-28) alone, 9.62 ± 0.30; with Gln Phe
Val Gly (SEQ ID NO: 2), 6.69 ± 0.22, $p < 0.01$; with Asp
Phe Phe Val Gly (SEQ ID NO: 1), 6.80 ± 0.38, $p < 0.01$;
and with Ala Ile Phe Thr (SEQ ID NO: 3), 6.92 ± 0.32,
$p < 0.01$.

-10-

Table 1

Effects of ICV co-administered non-amnestic
peptides on amnestic effects of ß-(12-28) on
retention of FAAT using groups of ten mice

Peptide	Trial to criterion, no (mean ± SEM)[1]	P-value for comparison with ß-(12-28) alone[3]
Vehicle alone	6.85±0.20	-
ß-(12-28) alone	9.62±0.30[2]	-
ß-(12-28) + Ala Val Phe	9.31±0.36	NS[4]
ß-(12-28) + Asp Phe Phe Val	9.31±0.38	NS
ß-(12-28) + Lys Leu Val Phe Phe	9.23±0.34	NS
ß-(12-28) + Asn Leu Ile Thr	9.15±0.41	NS
ß-(12-28) + Lys Leu Val Phe Phe Ala Glu	9.08±0.30	NS
ß-(12-28) + Phe Val Phe	8.92±0.26	NS
ß-(12-28) + Ala Phe Ile Gly	8.92±0.38	NS
ß-(12-28) + Val Val	8.85±0.42	NS
ß-(12-28) + Ser Phe Val Gly	8.85±0.41	NS
ß-(12-28) + Gly Phe Met Thr	8.85±0.47	NS
ß-(12-28) + Phe Phe	8.69±0.46	NS
ß-(12-28) + Ser Phe Phe Gly	8.08±0.40	NS
ß-(12-28) + Ala Ile Phe Thr	6.92±0.32	<0.01
ß-(12-28) + Asp Phe Phe Val Gly	6.80±0.38	<0.01
ß-(12-28) + Gln Phe Val Gly	6.69±0.22	<0.01

[1] The higher the mean the less the efficacy of a
peptide in blocking the amnestic effect of ß-(12-28).
[2] P<0.01 for comparison with vehicle alone.
[3] P values were obtained for selected comparisons
using Tukey's t-test after obtaining a significant F
value by analysis of variance (ANOVA).
[4] NS = not significant.

-11-

Subsequently Asp Phe Phe Val Gly (SEQ ID NO: 1)
and ß-(12-28) were given ICV separately post-training
before or after saline (2 μl each, 60 seconds apart)
or first Asp Phe Phe Val Gly (SEQ ID NO: 1) and then
ß-(12-28) or first ß-(12-28) and then Asp Phe Phe Val
Gly (SEQ ID NO: 1) (Figure 1). Whether saline was
given before or after ß-(12-28) did not affect the
result, indicating that increase of total volume
administered ICV from 2 μl to 4 μl did not matter.
The order of administration of ß-(12-28) and Asp Phe
Phe Val Gly (SEQ ID NO: 1) did not affect the ability
of the latter to block the amnestic effect of the
former (Figure 1). These latter results suggest, but
do not prove, that direct interaction of the
counter-amnestic peptides with ß-)12-28) is not the
reason for their protective action. Separate
experiments with the amnesia blockers Gln Phe Val Gly
(SEQ ID NO: 2), Asp Phe Phe Val Gly (SEQ ID NO: 1),
and Ala Ile Phe Thr (SEQ ID NO: 3) in weakly trained
animals (Table 2) showed these substances not to have
any memory-enhancing effects on retention of T-maze
FAAT, indicating that amnestic effects of ß-(12-28)
were not being overcome by independent
memory-enhancing effects of these substances.

Table 2

Effects of Asp Phe Phe val Gly (SEQ ID NO: 1),
Ala Ile Phe Thr (SEQ ID NO: 3) and Gln Phe Val
Gly (SEQ ID NO: 2) on retention of T-maze FAAT
measured in weakly trained mice (groups of 14 each)[1]

Peptide	Trial to criterion, no (mean ± SEM)	P-value for comparison with vehicle
Vehicle alone	9.07±0.25	–
Asp Phe Phe Val Gly	9.14±0.32	NS[2]
Ala Ile Phe Thr	9.43±0.30	NS
Gln Phe Val Gly	9.64±0.28	NS

-12-

1 This paradigm is designed to measure the extent of enhancement, if any, over that found with vehicle alone (0.001% DMSO in saline). None was observed.

2 NS = not significant.

Esters and Amides of Ala Ile Phe Thr (SEQ ID NO: 3), Asp Phe Phe Val Gly (SEQ ID NO: 1) and Gln Phe Val Gly (SEQ ID NO: 2) as Antagonists of Amnestic effects of Aβ. The most likely additional related substances to synthesize and administer would be esters and amides of the three active peptides (SEQ ID NOS: 1, 2 and 3) in which the carboxyl group of each of them is esterified or amidated.

The peptidic esters preferably have the structural formula:

$$\text{I.} \qquad X\!\!-\!\!\overset{\displaystyle \overset{O}{\parallel}}{C}\!\!-\!\!R$$

in which X is a peptide, SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 and R is a straight or branched chain alkyl group having one to eighteen carbon atoms, an aromatic group, e.g., a substituted or unsubstituted phenyl, napthyl or anthracyl group, a heterocyclic group, e.g., a pyridine or imidazale group or a steroidal group, e.g., pregnenolone, dehydroepiandosterone, progesterone or any biologically active steroid having an available hydroxyl group.

The peptidic amides have the structural formula:

$$\text{II.} \qquad X\!\!-\!\!\overset{\displaystyle \overset{O}{\parallel}}{C}\!\!-\!\!N\!\!\overset{\textstyle R_1}{\underset{\textstyle R_2}{<}}$$

in which X may be the same as X in Formula I and in which R_1 and R_2 are the same or different alkyl,

-13-

aromatic, heterocyclic or steroidal group as in
Formula I. Such substances may be more resistant to
enzymatic attack than the parent peptides and could
pass the blood-brain barrier more readily, whereupon
they would be hydrolyzed to form the effective
peptide in the brain.

-14-

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-16-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Eugene Roberts

    (ii) TITLE OF INVENTION: Method For
                                      Antagonizing Amnestic
                                      Effects of Amyloid ß
                                      Protein and Improving
                                      the Quality of Life
                                      in Individuals
                                      With Alzheimer Disease

    (iii) NUMBER OF SEQUENCES: 15

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    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: 3M Double Density 5
             1/4" diskette

        (B) COMPUTER: Wang PC

        (C) OPERATING SYSTEM: MS DOS Version 3.20

        (D) SOFTWARE: Microsoft

    (vi) CURRENT APPLICATION DATA:

        (A) APPLICATION NUMBER: Unknown

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-17-

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    (C)   TELEX: None

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 5

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

    Asp Phe Phe Val Gly
    1   5

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 4

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

    Gln Phe Val Gly
    1

-18-

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 4

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

        Ala Ile Phe Thr
        1

(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 2

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

        Phe Phe
        1

(2) INFORMATION FOR SEQ ID NO: 5:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 2

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

        Val Val
        1

-19-

(2) INFORMATION FOR SEQ ID NO: 6:

        (i) SEQUENCE CHARACTERISTICS:

             (A) LENGTH: 3

             (B) TYPE: Amino Acid

             (C) STRANDEDNESS:

             (D) TOPOLOGY: Unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

             Ala Val Phe
             1

(2) INFORMATION FOR SEQ ID NO: 7:

        (i) SEQUENCE CHARACTERISTICS:

             (A) LENGTH: 3

             (B) TYPE: Amino Acid

             (C) STRANDEDNESS:

             (D) TOPOLOGY: Unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

             Phe Val Phe
             1

(2) INFORMATION FOR SEQ ID NO: 8:

        (i) SEQUENCE CHARACTERISTICS:

             (A) LENGTH: 4

             (B) TYPE: Amino Acid

             (C) STRANDEDNESS:

             (D) TOPOLOGY: Unknown

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

             Ala Phe Ile Gly
             1

-20-

(2) INFORMATION FOR SEQ ID NO: 9:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 4

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

        Gly Phe Met Thr
        1

(2) INFORMATION FOR SEQ ID NO: 10:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 4

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

        Asn Leu Ile Thr
        1

(2) INFORMATION FOR SEQ ID NO: 11:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 4

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

        Ser Phe Phe Gly
        1

-21-

(2) INFORMATION FOR SEQ ID NO: 12:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 4

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

        Ser Phe Val Gly
        1

(2) INFORMATION FOR SEQ ID NO: 13:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 4

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

        Asp Phe Phe Val
        1

(2) INFORMATION FOR SEQ ID NO: 14:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 7

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

        Lys Leu Val Phe Phe Ala Glu
        1   5

-22-

(2) INFORMATION FOR SEQ ID NO: 15:

    (i) SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 5

        (B)   TYPE: Amino Acid

        (C)   STRANDEDNESS:

        (D)   TOPOLOGY: Unknown

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

        Lys Leu Val Phe Phe
         1   5

-23-

CLAIMS:

1. A peptide having the sequence of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

2. A method for antagonizing the amnestic effects of amyloid ß protein (Aß) which comprises administering to a mammal affected with the amnestic effects of Aß a therapeutically effective amount of a peptide having the sequence of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

3. A method as defined by claim 2 in which said peptide is administered orally, subcutaneously, intravenously, transdermally, intranasally, rectally, intrathecally, sublingually, or intracisternally.

4. A method as defined by claim 2 or claim 3 in which said mammal is a mouse.

5. A method as defined by claim 2 or claim 3 in which said mammal is a human.

6. An ester or an amide of a peptide as defined by claim 1.

7. A peptide ester having the structure Formula I.

8. A peptide amide having the structure Formula II.

Fig. 1.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00; C07K 5/00, 7/00, 17/00
US CL :530/330
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/330

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPTO APS
search terms: roberts, asp-phe-phe-val-gly, gln-phe-val-gly, ala-ile-phe-thr

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WYNGAARDEN ET AL, "CECIL TEXTBOOK OF MEDICINE", published 1992 by W. B. Saunders Company (Philadelphia, PA) pages 2076-2077.	1-8

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 NOVEMBER 1994	JAN 19 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SHEELA J. HUFF
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Form PCT/ISA/210 (second sheet)(July 1992)*

(54) **Title:** METHOD OF PREVENTING AGGREGATION OF AMYLOID β-PROTEIN

(57) **Abstract**

This invention is directed to methods and compositions for preventing aggregation of amyloid β-protein (βAP) aggregation. Aggregation of amyloid β-protein is associated with the deposition of amyloid in the brain. Amyloid β-protein-binding compounds such as transthyretin are described which form complexes with βAP and prevent formation of amyloid. This invention also identifies the serine 6 mutation in the TTR gene as predictive of person at risk for developing βAP associated amyloidosis.

- 1 -

METHOD OF PREVENTING AGGREGATION
OF AMYLOID ß-PROTEIN

TECHNICAL FIELD OF THE INVENTION

This invention relates to methods and compositions useful for preventing aggregation of amyloid β-protein. Aggregation of amyloid ß-protein is associated with development of amyloid deposits which are formed in persons with various forms of dementia. This invention also relates to the identification of a genetic marker useful for identifying persons at risk for developing amyloidosis. Accordingly, this invention relates to methods of diagnosing, preventing and treating amyloidosis associated with aggregation of amyloid ß-protein.

BACKGROUND OF THE INVENTION

The cardinal pathological feature of Alzheimer's disease is the formation of amyloid depositions of aggregated amyloid ß-protein (ßAP) in the brain and cerebralvasculature. Amyloidosis is a pathologic condition characterized by the deposition of amyloid, a generic term describing abnormal extracellular and/or intracellular deposits of fibrillar proteins. Amyloid deposits may be formed in a variety of tissue and organs including brain, liver, heart, kidney, etc. Advanced amyloidosis may cause extensive tissue breakdown.

Proteins involved in the formation of amyloid have the following common properties: 1) they possess a ß-pleated sheet secondary structure; 2) they form insoluble aggregates; 3) they exhibit green birefringence after

- 2 -

Congo red staining; and 4) they possess a characteristic
fibrillar structure when observed under an electron
microscopic. Proteins which have been identified as
capable of forming amyloid in human disease include:
immunoglobulin light chains, protein AA, β_2-microglobulin,
transthyretin, cystatin C variant, gelsolin,
procalcitonin, protease resistant protein PrP^{scr}, and
amyloid ß-protein.

Amyloid ß-protein, or βAP, a polypeptide of 39 to 43
amino acids is a 4-kilodalton derivative of a large
transmembrane glycoprotein amyloid ß precursor protein
(APP). See, D.L. Price, D.R. Borchelt and S.S. Sisodia,
Proc. Natl. Acad. Sci. U.S.A. **90**, 6381 (1993); Selkoe,
D.J. "Amyloid Protein and Alzheimer's Disease", <u>Scientific
American</u>, (1991) 265:68 for review. The sequence of
amyloid ß-protein was determined by Glenner and Wong,
Biochem. Biophys. Res. Comm. (1984) (120:885) and U.S.
Patent 4,666,829 which is incorporated herein by
reference. βAP is found in an aggregated, poorly soluble
form, in extracellular amyloid depositions in brains and
leptomeninges of patients with Alzheimer's disease (AD),
Down syndrome (DS), and hereditary cerebral hemorrhage
with amyloidosis - Dutch type (HCHWA-D) (D.J. Selkoe,
Neuron **6**, 487 (1991); D.L. Price, D.R. Borchelt and S.S.
Sisodia, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6381 (1993)).
In contrast, ßAP has been detected in a soluble form in
cerebral spinal fluid (CSF) and plasma of healthy
individuals and patients with AD. Seubert, P. et al.
<u>Nature</u> (1992), 359:325.

A number of studies of synthetic ßAP *in vitro* have
shown that ßAP aggregates easily and forms amyloid fibrils
similar to the fibrils found in brains of patients with AD
and DS (E.M. Castano et al., *Biochem. Biophys. Res.
Commun.* **141**, 782 (1986); D. Burdick et al., *J. Biol. Chem.*
267, 546 (1992); J.T. Jarrett and P.T. Lansbury, Jr.,
Cell, **73**, 1055 (1993)). The mechanism by which this

- 3 -

normally produced peptide forms amyloid is unknown. It
has also been unknown why ßAP, in a soluble form, is
present in biological fluids of healthy individuals and
patients with AD.

Recently, several extracellular proteins have been
identified that bind immobilized ßAP. These include apoE,
apoJ, and APP, all of which are found in CSF (W.J.
Strittmatter et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**,
8098 (1993); J. Ghiso et al., *Biochem. J.* **293**, 27 (1993);
W.J. Strittmatter et al., *Experimental Neurology* **122**, 327
(1993)). The binding of apoE, the major CSF
apoliproprotein which exists in 3 major isoforms, is
particularly relevant to late-onset familial and sporadic
AD. Patients homozygous for the apoE4 isoform have more
amyloid depositions than patients homozygous for the apoE3
isoform (W.J. Strittmatter et al., *Proc. Natl. Acad. Sci.
U.S.A.* (1993), 90:1977). The inheritance of the APOE4
allele also significantly increases the risk and decreases
the age of onset of AD (E.H. Corder et al., *Science* **261**,
921 (1993)). Although, apoE appears to be a candidate for
sequestration of ßAP, it was unknown as to whether apoE
bound ßAP and formed complexes *in vivo*.

Transthyretin (TTR), also referred to as prealbumin,
is a homotetrameric, protein each subunit of which
contains 127 amino acids. Its secondary, tertiary and
quartenary structure has been described Blake et al. in *J.
Mol. Biol.* (1978) 121:339, which is incorporated herein by
reference. The TTR tetramer has a molecular weight of
about 54,980 daltons. TTR is synthesized in liver and the
chorioid plexus and is present in the serum and cerebral
spinal fluid (CSF). In human CSF, TTR is usually present
at a concentration of about 0.3 micromolar. Only albumin
which is present in a concentration of about 2 micromolar
is present in CSF at a higher concentration. TTR is known
to be the main carrier of thyroxin and vitamin A across
the blood brain barrier. The presence of TTR in amyloid

- 4 -

deposits associated with AD and Down's syndrome has been
suggested by Shirahama, T. et al. Am. J. Pathol. (1982),
107:041, but not confirmed. Eikelenboom, P. and
F.C. Stam, Virchows Arch. [Cell P.] (1984), 47:17.

TTR which forms amyloid deposits in patients with
certain familial amyloid polyneuropathy's (FAP's) has been
determined to have various amino acid substitutions
compared to circulating transthyretin of normal
individuals. For example, a substitution of a methionine
for valine at amino acid residue number 30 has been
identified in kinships of Portuguese (Saraiva, M.J.M. et
al., J. Clin. Invest (1984) 74:104), Japanese (Tawara, S.
et al., Biochem. Biophys. Res. Comm. (1983) 116:880) and
Swedish ancestry (Dwulet, et al., Proc Natl. Acad. Sci.
USA (1984) 81:694; and Whitehead, A.S. et al., Mol. Biol.
Med. (1985) Vol. 7). In another form of FAP disease, the
TTR protein present in the affected individuals has serine
substituted for isoleucine at position 84. Wallace et
al., Clin. Res. (1985) (33:592A). Studies of TTR levels
in AD patients and patients with Down syndrome report that
TTR concentrations may be decreased in these patients.
Riisoen, H., Acta Neurol. Scand. (1988) 78:455 and
Elovaara, I. et al., Acta Neurol. Scand. (1986) 74:245.

Sipe et al., United States Patent 4,186,388 refers to
the cloning of the human TTR gene and its use to identify
various forms of FAP's. Use of the gene and specific cDNA
fragments capable of hybridizing with DNA fragments of
biological samples is reported to be useful to identify
individuals with various forms of FAP disease including
type I FAP disease in which methionine is substituted for
valine at position 30 as described above. Sipe et al.
report that TTR is associated with amyloid deposits in
Alzheimers disease, FAP, and senile cardiac amyloidosis.
Sipe et al., further state that the function of TTR in the
nervous system is unknown.

- 5 -

Chromosomal localization of genes causing AD can facilitate early diagnosis of persons with this disease. Prenatal diagnosis in affected families is also possible once a genetic marker for a disease is identified. Subsequent delineation of closely linked markers which show strong linkage disequilibrium with the disorder and ultimately, identification of the defective gene can allow screening of the entire at-risk population to identify carriers, begin early prophylactic or therapeutic invention if available and potentially reduce the incidence of new cases.

There is a need for effective methods and compositions for preventing aggregation of ßAP and of identifying individuals at risk for developing amyloidosis.

SUMMARY OF THE INVENTION

This invention provides methods and compositions useful for preventing aggregation of amyloid ß-protein. The methods are useful for preventing aggregation of amyloid ß-protein (ßAP) *in vivo* and *in vitro* and therefore, may be used to prevent or treat mammals, especially humans with amyloidosis associated with ßAP aggregation. The methods are also useful for diagnosing persons at risk for developing amyloidosis associated with amyloid ß-protein aggregation.

The compositions of this invention promote complex formation between ßAP and ßAP-binding compounds such as TTR, which are capable of complexing with ßAP, in a manner which prevents ßAP from self-aggregating and forming amyloid.

The method of preventing aggregation of ßAP according to this invention comprises providing a ßAP-binding compound to a fluid or biological tissue comprising ßAP. The ßAP-bind compound is provided in an amount sufficient

- 6 -

to sequester ßAP in complexes comprising ßAP and the ßAP-binding compound so that ßAP is not available to self-aggregate.

By preventing ßAP aggregation and amyloid deposition, this invention also provides methods and compositions useful for preventing and treating diseases associated with ßAP amyloid formation, including, for example, AD, Down's Syndrome and hereditary cerebral hemorrhage with amyloidosis - Dutch type.

This invention also provides assays for detecting ßAP or TTR in a biological fluid based on the formation of complexes comprising ßAP and at least one ßAP-binding compound.

Also provided is a method of detecting persons at risk for developing ßAP associated amyloidosis by identifying the presence of a mutation in the TTR gene. The mutation involves a G-A transition in codon 6 resulting in the substitution of serine at amino acid position 6 for the normally present glycine. Because this mutation also creates a BsrI restriction site, this invention also provides a method of identifying persons at risk for developing ßAP amyloidosis. The method comprises obtaining DNA from a person; amplifying the gene, or portion thereof, comprising the codon for the sixth amino acid (glycine) of TTR; digesting the amplified DNA with a restriction enzyme such as BsrI; and analyzing the fragments for the presence of an alteration in restriction fragments compared to controls to detect the Serine 6 mutation.

It is an object to this invention to provide methods of preventing aggregation of ßAP in solution by sequestering ßAP in complexes with ßAP-binding compounds such as TTR. Prevention of aggregation is useful for preventing fibril and amyloid formation associated with disease.

- 7 -

Another object of this invention is to provide compositions useful for complexing with ßAP to so as to sequester ßAP preventing ßAP from forming aggregates.

It is another object to this invention to provide assays suitable for determining the amount of ßAP or TTR in a biological fluid.

Another object of this invention is to provide methods and compositions useful for genetic screening of individuals to identify individuals who have a mutation in the TTR gene and who may be at risk for developing ßAP associated amyloidosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Analysis of ^{125}I-ß complexes with CSF proteins. $10\mu l$ CSF were incubated with 10^5 dpm ^{125}I-ßAP$_{1-28}$ (specific activity 3-6 x 10^6 dpm/μg) in a final volume of $20\mu l$ PBS, Ph 7.4 at 37°C for 8 hours, except an experiment illustrated in Figure 1a. The complexes were analyzed by electrophoresis in a 12% SDS-polyacrylamide gel under non-reducing conditions except for an experiment illustrated in figure 1g, lane 2.

a.   Comparison of complexes of ^{125}I-Aß$_{1-28}$ with ApoE3 (1.5 μM) (lane 1) and with CSF proteins (lane 2) formed after incubation at 37°C for 24 hours.

b.   Time course of complex formation of ^{125}I-Aß$_{1-28}$ with CSF proteins.

c.   Time course of complex formation of ^{125}I-Aß$_{1-28}$ with CSF proteins in presence of 1.5 μM human plasma ApoE3.

d.   Competition of complex formation of ^{125}I-Aß$_{1-28}$ with CSF proteins by unlabeled Aß$_{1-28}$.

CSF (lane 1), CSF plus 10 fold excess of unlabeled Aß$_{1-28}$ (lane 2), CSF plus 200 fold excess of unlabeled Aß$_{1-28}$.

e.   Competition of complex formation of ^{125}I-Aß$_{1-40}$ with CSF proteins by unlabeled Aß$_{1-40}$.

- 8 -

CSF (lane 1), CSF plus 10 fold excess of unlabeled $A\beta_{1-40}$ (lane 2), CSF plus 200 fold excess of unlabeled $A\beta_{1-40}$.

f.   Competition of complex formation of $^{125}I\text{-}A\beta_{1-40}$ with TTR by unlabeled $A\beta_{21-40}$.

0.1 μM TTR (lane 1),

0.1 μM TTR plus 100 fold excess of unlabeled $A\beta_{1-40}$ (lane 2),

0.1 μM TTR plus 500 fold excess of unlabeled $A\beta_{1-40}$ (lane 3).

g.   Analysis of complexes of $^{125}I\text{-}A\beta_{1-28}$ with TTR under different conditions.   Before electrophoresis the samples were incubated in 100 mM Tris-HCl, pH 6.8 without ß-mercaptoethanol for 5 minutes at room temperature (lane 1), or were boiled for 10 minutes in a complete Laemli buffer with 0.2 M ß-mercaptoethanol (lane 2).

Figure 2. $^{125}I\text{-}\beta AP$ complexes with CSF proteins.

a.   SDS-PAGE analysis of $^{125}I\text{-}\beta AP_{1-28}$ incubated for 24 hrs. in PBS (lane 1) and after centrifugation through a 20% sucrose cushion at 15000 x g for 10 minutes (lane 2).

b.   SDS-PAGE analysis complexes of $^{125}I\text{-}\beta AP_{1-28}$ with CSF proteins formed after incubation for 24 hrs. (lane 1) or 10 minutes (lane 2); or after incubation with human apoE3 in PBS after 24 hrs. (lane 3).

Figure 3.   Western blot analysis of TTR-ßAP complexes.

a. βAP_{1-40} (10 μg) incubated overnight at 37°C in PBS, pH 7.2 with CSF (10 μl, lane 1); TTR (1 μg, lane 2); BSA (50 μg, lane 3).   Control consisted of CSF (10 μl, lane 4) and TTR (1 μg, lane 5) without ßAP.   Samples were analyzed by SDS-PAGE and immunoblotting with rabbit anti-ßAP antibody SGY2134.

b.   Immunoblotting with rabbit anti-ßAP antibody (SGY2134, lane 1) and sheep anti-TTR (lane 2) of βAP_{1-28} and CSF (10 μl) overnight incubation in PBS, pH 7.2 at

- 9 -

37°C analyzed by SDS-PAGE and immunoblotting. The immunoblot was cut lengthwise in two strip for analysis in lanes 1 and 2.

Figure 4.   Prevention of aggregation of ßAP.

a. ^{125}I-ßAP aggregates.

b.   Effect of TTR on aggregation of ^{125}I-ßAP.

c.   Thioflavin T based fluorometric assay of ßAP$_{128}$ aggregation in the presence of different concentrations of borine serum albumin (x), TTR (□), apoE3 (Δ), and apoE4 (O). Each point represents the average of quadruplicate measurements and are plotted as percentages with standard error for the given concentrations.

Figure 5.   Congo red staining of ßAP$_{128}$ aggregates in the presence of 5 μM BSA (right panel) or 3 μM TTR (left panel). Slides were viewed under polarized light at 200 x magnification.

Figure 6.   Electron micrograph of ßAP$_{128}$ aggregates without (right panel) or with 2 μM TTR (left panel). Scale bar, 100 nm. Samples were examined and photographed at magnification of 25,000 in a Hitachi-12 electron microscope.

Figure 7.   Computer graphic models of ßAP (top), TTR dimer (middle) and TTR-ßAP complex (bottom).

Figure 8.   Serine 6 polymorphism. Agarose gel electrophoresis of a DNA fragment corresponding to exon 2 of TTR gene amplified by PCR and analyzed for the presence of the BsrI restriction site in individuals who are normal (lanes 2 and 3), homozygous (lane 4) and heterzygons (lanes 5 and 6) for the serine 6 mutation. Lanes 1 and 7 are <u>Hae</u> III digests of ϕ x 174 DNA for use as molecular weight markers.

- 10 -

DETAILED DESCRIPTION OF THE INVENTION

This invention describes methods and compositions
useful for preventing aggregation of soluble amyloid
ß-protein. We have determined that ßAP present in
cerebral spinal fluid (CSF) is predominantly bound to TTR.
In addition, we have identified a unique binding
interaction between TTR and ßAP in biological fluids. In
another embodiment of this invention a mutation in the
gene encoding transthyretin has been identified in
patients with AD, the presence of which is highly
predictive of patients at risk for developing AD and is
also useful for diagnosing persons with AD.

This invention provides compounds useful for
preventing aggregation of ßAP in solution. We have
identified that ßAP which normally self-aggregates when in
solution, may be prevented from self-aggregating by
causing the ßAP to form a complex with ßAP-binding
compounds (BBC's). For the purposes of this invention,
BBC's are any compound which form a complex with ßAP and
which prevent ßAP aggregation and formation of amyloid.
Examples of BBC's for use with this invention include, but
are not limited to, TTR, TTR analogs, and apoE.

The identification of the ability of BBC's to prevent
aggregation of ßAP, and subsequent amyloid formation,
provides for methods of diagnosing, preventing, and
treating persons with, or at risk for developing
amyloidosis resulting from ßAP aggregation.

Binding of a BBC to ßAP occurs at physiologically
relevant concentrations. For example, TTR which is
normally present in CSF at a concentration of about 300 nM
may be caused to bind to ßAP present in a solution at
concentrations of about 3 nM, which is similar to ßAP's
concentration in CSF.

By assessing the binding interaction between a BBC,
such as for example TTR, and ßAP in an appropriate fluid

- 11 -

or biological tissue including CSF, one can monitor the
extent of BBC-ßAP complex formation. Such monitoring of
BBC-ßAP complex formation may be done in the context of a
diagnostic test to determine whether the normal BBC-ßAP
binding phenomenon is altered.

5   Alterations in BBC-ßAP complex formation *in vivo*
resulting from either alterations in BBC's or ßAP may
result in increased amounts of free ßAP available to
aggregate and form amyloid. Changes in the amino acid
sequence of BBC's or ßAP, or in their rate of synthesis or
10   degradation may result in a decrease in complex formation
resulting in ßAP aggregation. Such alterations would put
a person at risk for developing amyloid deposits resulting
from ßAP aggregation.

        We have determined that TTR is the predominant BBC in
15   CSF which is responsible for preventing ßAP from
aggregating and forming amyloid. In CSF, TTR binds with
ßAP to form a complex which sequesters ßAP and prevents
ßAP from self-aggregating. Because TTR is the predominant
BBC in CSF, to determine if someone is at risk for
20   developing amyloidosis resulting from ßAP aggregation, it
would be preferable to monitor the binding interaction
between ßAP and TTR. Binding of TTR to ßAP may also be
monitored to determine the effectiveness of a treatment
directed at sequestering ßAP or reducing the concentration
25   of ßAP available for aggregation. Measuring TTR-ßAP
binding interactions may also be used to identify other
BBC's which are also suitable for complexing to ßAP in a
manner which sequesters ßAP so that it is unavailable to
aggregate and form amyloid depositions.

30   Binding of BBC to ßAP may be measured by using
standard binding assays known to those skilled in the art,
based on the teachings disclosed herein. For example, ßAP
and TTR may be allowed to form complexes in solution.
Detection of the complexes may be accomplished using
35   chromatographic techniques, for example, molecular sieve

- 12 -

exclusion chromatography. SDS-PAGE electrophoresis may also be used.

Assays involving immobilization of either ßAP or TTR to a solid support may also be used to detect binding of the corresponding partner (i.e., TTR if ßAP is immobilized). The method of Strittmatter, W. et al., *Proc. Natl. Acad. Sci.*, *USA* (1993), 90:1977 which is incorporated herein by reference, for binding apoliprotein (APOE) to ßAP, may be used to demonstrate complex formation between TTR and ßAP. Detection of bound TTR or ßAP may be accomplished by methods well known in the art including use of enzymatic or radioactive labels.

Binding of human TTR to ßAP is specific. Evidence of specific binding between TTR and ßAP may be provided using a competition assay. In such an assay, either one of ßAP or TTR is labelled in a binding assay as described above. An excess of unlabelled ligand of the same type which is labelled (i.e., excess of ßAP if labelled ßAP is used) is included in the reaction mixture to compete with and prevent binding of the labelled ligand. Preferably, the unlabelled ligand will be present in the reaction mixture in excess of between 100 to 1000 times the concentration of the labelled ligand.

BBC's which are useful with this invention prevent the aggregation of ßAP and subsequent amyloid formation. The inhibition by a BBC of one or more properties of amyloid, would be indicative of a BBC useful with this invention. These properties of amyloid include, for example, the ability of a BBC to 1) prevent ßAP from assuming a ß-sheet secondary structure, 2) prevent ßAP from aggregating to form an insoluble structure, 3) inhibit ßAP from forming structures which exhibit green birefringence after Congo red staining, and 4) prevent ßAP from forming fibrils with typical electron microscopic appearance. TTR, which is a preferred BBC for use with

- 13 -

this invention, prevents amyloid formation according to
all four criteria.

    We used computer modeling of the binding interaction
between ßAP and TTR to identify the specific molecular
surfaces of ßAP and TTR which participate in complex
formation. The identification of the amino acid sequences
of TTR and ßAP relevant to the binding interaction allows
for the synthesis of other BBC's including polypeptides,
analogs of TTR and other molecules which would bind to ßAP
to prevent its aggregation. Analogs of TTR include
fragments of TTR, and TTR having amino acid substitutions,
deletions, or additions as well as other molecules that
share a similar TTR binding domain which is important for
preventing amyloid formation. The TTR analogs form
complexes with ßAP to prevent its aggregation and amyloid
formation. In addition, unless otherwise specifically
stated, reference herein to TTR is meant to include TTR
analogs as well. Based on the teachings disclosed herein,
other types of molecules may also be capable of binding to
ßAP to prevent its aggregation. Such molecules include
organic molecules such as for example
Buckministerfullerenes. Preferably, BBC's and TTR analogs
bind to ßAP at concentrations which avoid harmful side
effects in an individual to whom they may be administered.

    The TTR analogs may include analogs of TTR in which
the amino acid sequence of human TTR is substituted with
amino acids which allow for the TTR-ßAP binding
interaction to occur. Such substitutions include
substituting neutral amino acids such as glycine, alanine,
valine, leucine and isoleucine for other neutral amino
acids present in the TTR sequence. In addition,
substitution of aromatic amino acids present in TTR may be
accomplished using phenylalanine, tyrosine or tryptophan.
Aliphatic amino acids in TTR possessing hydroxyl groups
may be substituted with serine or threonine. Amino acids
present in TTR having basic side chains may be substituted

- 14 -

with lysine, arginine, and histidine, whereas amino acids having acidic side chains may be substituted with aspartic acid or glutamic acid. Asparagine may be substituted for glutamine which both have amide chains.

Because of a large contact surface between ßAP and TTR it may be possible that not all amino acid substitutions be a precise one-to-one equivalency. Some flexibility of the choices may be necessary, as long as the analog attempts to reproduce with reasonable fidelity the shape of the TTR binding surface and the engendered electrostatic potential mimics that of TTR within a reasonable range. More precisely the -1kT electrostatic potential contour of the analog should cover essentially the same area as in TTR and its location should not be closer than about a 2Å displacement toward the surface, as compared with the TTR potential contour.

Without being bound by theory, the portion of the amino acid sequence of TTR which participates in the binding with ßAP extends from about amino acid residue 30 to 70. Within this amino acid sequence, the specific amino acids of TTR comprising Arg (34, 161), Ala (37, 164), Asp (38, 165), Thr (40, 167), Glu (42, 169), Glu, (62, 189), Val (65, 192), and Glu (66, 193) are preferred. (The first number is the order number at the first residue on chains 1 and 3; the second number refers to the residue in chains 3 and 4.)

According to the model, negative amino acid residues Asp (38, 165), Glu (42, 169), Glu (62, 189), and Glu (66, 193) present on the essentially convex surface of TTR comprising by all the amino acids specified in the preceding paragraph are responsible for generating a negative electrostatic potential around that specific region of TTR so as to specifically interact with the positive electrostatic potential engendered by specific positive amino acids on the surface of the ßAP. These positive amino acids of ßAP, which are located in an

- 15 -

essentially concave surface which specifically interacts
with TTR to form the complex include Arg (5), His (13),
Lys (16), and Lys (28). These positive amino acids on ßAP
reside in the essentially concave larger contacting
surface of ßAP referred to above comprising amino acid
residues Arg (5), Ser (8), Gly (9), Val (12), His (13),
Gln (15), Lys (16), Phe (19), Phe (20), Asp (23), Val
(24), Asn (27), and Lys (28).

The most important parameters for describing the
binding interaction between TTR and ßAP are the detailed
curvatures of the surface and the electrostatic potentials
generated by the charged amino acids. The contacting
surfaces of TTR and ßAP are the surfaces described by the
listing of the specific amino acid residues in TTR and ßAP
listed above. The negative electrostatic potential on TTR
is engendered by the negative amino acids listed above,
and similarly, the positive amino acids on ßAP engender
the positive potential around its surface.

This invention includes other BBC's which bind to the
TTR binding site of ßAP, or portions thereof, and which
prevent the aggregation of ßAP and formation of amyloid.
In addition, compounds which alter the TTR binding site of
ßAP, whether or not they bind to this site, but bind to
ßAP and prevent its aggregation are contemplated as well.
Also, compounds that possess a surface shape and
electrical change similar to the binding site of TTR or
part thereof, could prevent ßAP aggregation and amyloid
formation and are contemplated as well.

This invention provides a method of preventing
aggregation of s⊂ ble ßAP present in a solution at a
given concentrati⸱⸱⸱. ßAP may be present in solutions *in
vitro* or *in vivo*. Biological tissues in which ßAP is
present include CSF, cerebralvasculature, or brain. To
prevent aggregation of ßAP, a sufficient amount of TTR or
TTR analog is provided to the ßAP containing solution or
tissue to form TTR-ßAP complexes. As shown in Fig. 6,

- 16 -

evidence of inhibition of aggregation may be demonstrated
by the inhibition of ßAP-fibril formation.

Inhibition of ßAP may be accomplished over a wide BBC
to ßAP ratio. In CSF of normal individuals in which ßAP
is not aggregated, TTR is present at a concentration of
about 300 nM, whereas ßAP is present at about only 3 nM.
Accordingly, a suitable ratio of TTR to ßAP for use with
this invention is about 100 to 1. However, *in vitro*, we
have determined that TTR, at about 1.2 μM, prevents about
50% of aggregation of ßAP present at about 300 μM. Fig.
4. Therefore, a ratio of BBC to ßAP of about 1 to 100 is
also preferred for use with this invention. In addition,
a ratio of BBC to ßAP of about 1 to 4 is also preferred
based on the stoichiometry of the ßAP and TTR binding.
BBC to ßAP ratios of 1 to 2 and 1 to 1 are also preferred.
These ratios may be optimized for use with BBC's besides
TTR.

As the concentration of TTR is increased, the
formation of ßAP aggregates having a high molecular weight
decreases. Fig. 4(b). Because as little as about 3 μM TTR
can essentially completely inhibit aggregate formation of
about 300 μM ßAP, it is likely that TTR also inhibits
aggregation of ßAP by mechanisms other than by the simple
stoichiometric model discussed above. Accordingly, the
administration of BBC's such as TTR would be useful to
prevent the enlargement of amyloid deposits existing prior
to initiation of treatment.

Endogenous TTR present *in vitro* or *in vivo*, which is
capable of binding to ßAP at the TTR binding site in a
manner which prevents ßAP aggregation, for the purpose of
this invention is considered to contribute to the total
amount of TTR or TTR analog in determining the ratios
described above. In situations were endogenous TTR is
mutated or is in a form which does not effectively prevent
ßAP aggregation, then the amount of exogenous TTR, or TTR
analog, added to the solution would be an amount

- 17 -

sufficient to inhibit ßAP aggregation independent of the
endogenous TTR and preferably would be an amount to
achieve one of the ratios described above.

The TTR-ßAP binding interaction described according
to the invention is also useful in assays to determine the
amount of ßAP or TTR in a biological sample. Several
protocols known in the art including immunoassay and
receptor binding assays may be adapted to take advantage
of the complex formation formed between ßAP and TTR. For
example, according to one embodiment of this invention, an
assay to determine the amount of soluble ßAP present in a
sample would comprise the steps of combining the sample
with a soluble or bound BBC, such as TTR, in the presence
of a known amount of soluble labelled ßAP and detecting
the amount of ßAP in the sample. According to a preferred
embodiment, TTR is bound to a solid support.

In another embodiment of the assay, a sample
containing an unknown amount of TTR or mutated form of TTR
may be combined with ßAP bound to a solid support.
Labeled TTR may then be added to the sample to determine
by competition the amount of endogenous TTR or mutated TTR
present in the sample.

Other assay protocols including sandwich assays are
contemplated by this invention. For example, to measure
ßAP in a sample, TTR may be linked to an insoluble support
to which is added the sample containing the unknown amount
of ßAP. Anti-ßAP-antibody which is labeled may then be
added to the sample to detect the amount of bound ßAP.
Such an assay may also be constructed to determine the
amount of TTR in a sample.

In another embodiment of the invention, soluble ßAP
in vivo is prevented from aggregating by providing a BBC
to form complexes with ßAP in vivo. The BBC, preferably
TTR, is provided in an amount sufficient to complex with
soluble ßAP so as to reduce the concentration of free ßAP
in solution. Reduction of soluble ßAP and the formation

- 18 -

of BBC-ßAP complexes sequesters the ßAP and decreases the
amount of ßAP available for forming aggregates and amyloid
deposition.   Inhibition of ßAP aggregation and amyloid
formation is useful from the prevention or treatment of
Alzheimer's Disease, Down's Syndrome and hereditary
cerebral hemorrhage with amyloidosis - Dutch type.

     The methods and compositions of this invention are
also suitable for use with mammals besides humans such as
monkeys, dogs and any other mammal that develops ßAP
amyloidosis.   (D.J. Selkoe, *Neuron* 6, 487 (1991).

     This invention also provides a method of identifying
persons at risk for developing amyloidosis based on the
identification of a mutation in the TTR gene involving a
substitution of serine for glycine at position 6 of TTR.
This substitution arises from a single point mutation in
which the first guanine in the GGT codon is substituted
with an adenine to produce the AGT codon.   A study by
Jacobsen et al. "Transthyretin ser 6 gene frequency in
individuals without amyloidosis", VII International
Symposium on Amyloidosis, July 11-15, 1993, 100, which is
incorporated herein by reference, reports that the serine
6 gene is a common normal TTR polymorphism present at a
frequency of about 12 % and, "apparently not associated
with amyloidosis in the Caucasian population." We have
surprisingly found that in a population of 55 unrelated AD
patients, 10 (i.e. 18 %) were heterozygous for this serine
6 mutation.   As AD is believed to be a heterogenous
disorder arising from a variety of causes, this result is
consistent with the serine 6 mutation identifying a
subpopulation of AD patients.   This mutation was found in
7 families with patients with late onset of AD disease.

     To identify persons at risk of developing AD
associated with the serine 6 mutation, the DNA containing
the second exon encoding TTR may be sequenced by methods
known to those skilled in the art, or the DNA may be
analyzed using restriction enzymes which can identify a

- 19 -

change in the recognition site for the restriction enzyme.
The substitution of A for G, destroys an <u>Msp</u>I site and
creates a <u>Bsr</u>I site. The methods described in Sipe et al.
U.S. patent 4,816,388, which is incorporated herein by
reference, may also be modified to identify the serine 6
mutation.

In one method, PCR-SSCP analysis as described by
Orita et al., <u>Genomic</u>, (1989), 5:874-879, which is
incorporated herein by reference, is performed to identify
the serine 6 mutation. Using PCR, primers which amplify
the second exon of TTR may be used to amplify the region
of the gene containing the serine 6 mutation. PCR may be
performed using methods described in Mullis et al. U.S.
patent 4,683,195, which is incorporated herein by
reference. The following oligonucleotide probes are
suitable for amplifying the appropriate region of exon 2
of TTR:

5' CGC TCC AGA TTT CTA ATA CCAC 3'   (1515-1537)
5' AGT GAG GGG CAA ACG GGA AGAT 3'   (1791-1769)

(The number in parenthesis represents the positions of the
bases in the TTR gene.)

Following amplification of the gene fragment, the
fragment may be sequenced or treated with restriction
enzymes to determine whether the serine 6 mutation is
present. Following separation of the amplified DNA
fragment from the genomic DNA, the amplified fragment may
be digested with BsrI to determine if this restriction
site is present.

The identification of a marker associated with a form
of AD lends itself to the formulation of kits which can be
utilized in diagnosis. Such a kit may comprise a carrier
being compartmentalized to receive in close confinement
one or more containers wherein a first container may
contain oliogonucleotides for amplifying the appropriate
region of the genomic DNA. Other containers may contain
reagents, such as restriction enzymes or labelled probes,

- 20 -

useful in the detection of the mutation. Still other
containers may contain buffers and the like.

BBC's such as TTR, and in particular, analogs
suitable for use in this invention, may be produced by
means known in the art including linking of individual
amino acids to construct specific sequences, modification
of purified TTR or by recombinant techniques. Recombinant
production of TTR is described in Sipe et al., United
States Patent No. 4,816,388 which is incorporated herein
by reference. To produce TTR analogs, cDNA encoding TTR
may be modified to contain coding sequences coding for the
desired TTR analog. Standard synthetic chemical
techniques may be used for producing other BBC's.

To provide treatment, or prevent amyloidosis
associated with ßAP aggregation, BBC's such as TTR, should
be administered to the individual in need of treatment in
a therapeutically effective amount. Preferably, the BBC
should be administered to the individual in an amount
sufficient to achieve a concentration *in vivo* sufficient
to prevent aggregation of ßAP.

BBC's such as TTR, which are to be administered
according to this invention may be administered as a
pharmaceutical composition further comprising a
biologically acceptable carrier including, but not limited
to, saline, buffer, dextrose and water.

BBC's such as TTR may be administered by known
methods including, sublingual, intravenous,
intraperitoneal, percutaneous or intranasal modes of
administration. Local administration directly to the site
of action may also be desirable and may be accomplished
through means known in the art including, but not limited
to, injection, infusion and implantation of infusion
devices containing the BBC. Similarly to administration
of other peptides, administration is preferably by means
which avoid contact with the gastrointestinal tract. The
administration of a BBC such as TTR directly to the CSF

- 21 -

may be accomplished by intrathecal injection. In a
preferred method of the invention, BBC, and in particular
TTR, is provided to the individual in need of treatment by
inducing its endogenous production in the individual in
need of treatment.

In another embodiment of the invention, a BBC such as
TTR may be provided to an individual through gene therapy.
To provide gene therapy to an individual, specific DNA
sequences which code and express a desired protein are
inserted into an appropriate vector complex which is then
used to infect an individual in need of treatment.
Various methods and vectors may be used for introducing a
desired genetic sequence into an individual. The
preferred and most often used method, incorporates the
desired genetic sequence, for example a cDNA encoding TTR,
into the genome of a retrovirus to form chimeric genetic
material. The genetically altered retrovirus may then be
used to infect the appropriate target cells *in vitro* or *in
vivo*. Preferably, the retrovirus is altered so the
desired sequences are inserted into the genome of the
target host cells and replicated without replicating the
infecting virus. The result of a successful gene transfer
via a retrovirus vector is a virally infected host cell
which expresses only the desired gene product. For
reviews on gene therapy using retroviral vectors see WO
92/07943 published May 14, 1992 "Retroviral Vectors Useful
for Gene Therapy" and Richard C. Mulligan, "Gene Transfer
and Gene Therapy: Principle, Prospects and Perspective" in
Etiology of Human Disease at the DNA Level, Chapter 12.
Jan Linsten and Alf Peterson, eds. Rover Press, 1991,
which are incorporated herein by reference. Additional
viral vectors suitable for providing gene sequences
include but are not limited to adeno-associated viruses,
Herpes Simplex 1 Virus and vaccinia.

The biological activity of ßAP has been demonstrated
in a number of experiments (1, 16). Binding of TTR and

- 22 -

other proteins to ßAP may regulate its biological activity
and play a role in the transport of the peptide.

The identification of ßAP binding proteins suggests
that prevention of ßAP aggregation and amyloid formation
requires a dynamic equilibrium of multiple extracellular
factors participating in the sequestration of ßAP. A
decreased level of TTR in CSF (H. Riisoen, *Acta Neurol.
Scand.* **78**, 455 (1988); I. Elovaara, C.P.J. Maury, J. Palo,
Acta Neurol. Scand. **74**, 245 (1986)) and an increased
expression of apoE, apoJ, and APP in the brains of AD
patients (J.F. Diedrich et al., *J. Virol.* **65**, 4759 (1991);
D.L. Price, D.R. Borchelt and S.S. Sisodia, *Proc. Natl.
Acad. Sci. U.S.A.* **90**, 6381 (1993)). P.C. May et al.,
Neuron **5**, 831 (1990) could alter the existing equilibrium
and facilitate amyloid formation. Subtle differences,
such as the single amino-acid substitution previously
demonstrated for two apoE isoforms, may significantly
influence the amount of amyloid formed in AD brains (D.E.
Schmechel et al., *Proc. Natl. Acad. Sci. U.S.A.* (1993),
90:9649-9653). Over thirty mutations have been documented
in TTR, and some lead to TTR amyloid formation in familial
amyloidotic polyneuropathy (M.D. Benson and M.R. Wallace
in *The Metabolic Basis of Inherited Disease*, C.R. Scriver,
A.L. Beudet, W.S. Sly and D. Valle Eds. (McGraw-Hill Book
Co., New York, 1989), pp. 2439-2460; D.R. Jacobson and
J.N. Buxbaum in *Advances in Human Genetics* Vol. 20, H.
Harris and K. Hirschhorn Eds. (Plenum Press, New York,
1991), pp. 69-123). Variants of TTR could be associated
with AD in families not linked to other defined genetic
loci on chromosomes 14, 19 and 21 (D.J. Selkoe, *Neuron* **6**,
487 (1991); D.L. Price, D.R. Borchelt and S.S. Sisodia,
Prox. Natl. Acad. Sci. U.S.A. **90**, 6381 (1993); E.M.
Castano and B. Frangione, *Lab. Invest.* **58**, 122 (1988)) or
modulate the effect of the defined loci. The suggested
structure of TTR-ßAP complex, furthermore, provides a

- 23 -

molecular basis for the design of drugs to prevent amyloid formation.

EXAMPLES

Example 1

A.   To identify the proteins interacting with ßAP in human CSF, synthetic $\text{ßAP}_{1\text{-}28}$ and $\text{ßAP}_{1\text{-}40}$ labeled by an iodinated Bolton-Hunter reagent were used.

Synthetic $\text{ßAP}_{1\text{-}28}$ and $\text{ßAP}_{1\text{-}40}$ from Bachem were radioiodinated using ^{125}I Bolton-Hunter reagent from Amersham according to manufacturer's instructions.   Ten microliters of human CSF were incubated with 10^5 dpm $^{125}\text{I-}$ $\text{ßAP}_{1\text{-}28}$ (specific activity 3-6 x 10^6 dpm/μg) in a final volume 20μl PBS, pH 7.4 at 37°C hours.   Incubation under five (5) different conditions (A, B, C, D and E) were conducted to analyze complex formation of ßAP.   The incubation conditions were as follows:

(A) $^{125}\text{I-ßAP}_{1\text{-}28}$ was incubated for 24 hours in PBS (Fig. 2(a), lane 1). $^{125}\text{I-ßAP}_{1\text{-}28}$ was incubated for 24 hours in PBS, centrifuged through 20% sucrose cushion at 15000xg for 10 minutes and the pellet was analyzed by SDS-PAGE (Fig. 2(a), lane 2).

(B)   Complexes of $^{125}\text{I-ßAP}_{1\text{-}28}$ with CSF proteins formed after incubation for 24 hours (Fig. 2(b), lane 1) or 10 minutes (Fig. 2(b), lane 2).   Complexes of $^{125}\text{I-ßAP}_{1\text{-}28}$ with human apoE3 formed after incubation for 24 hours (Fig. 2(b), lane 3).

(C)   Competition of complex formation of $^{125}\text{I-ßAP}_{1\text{-}28}$ with CSF proteins by unlabeled $\text{ßAP}_{1\text{-}28}$.   Radiolabeled ßAP was incubated with CSF (Fig. 1(d), lane 1), with CSF and 10 fold excess of unlabeled $\text{ßAP}_{1\text{-}28}$ (Fig. 1(d), lane 2), with CSF and 200 fold excess of unlabeled $\text{ßAP}_{1\text{-}28}$.   A triangle indicates a 30 kDa band.

(D)   Competition of complex formation of $^{125}\text{I-ßAP}_{1\text{-}28}$ with TTR (Calbiochem) by unlabeled $\text{ßAP}_{1\text{-}40}$.   Radiolabeled

- 24 -

ßAP was incubated with 0.1 μM TTR (Fig. 1(f), lane 1), 0.1 μM TTR and 100 fold excess of unlabeled ßAP$_{1-40}$ (lane 2), 0.1 μM TTR and 500 fold excess of unlabeled ßAP$_{1-40}$ (lane 3).

(E)   Effect of boiling in SDS under reducing conditions on TTR-ßAP complexes. ^{125}I-ßAP$_{1-28}$ was incubated with 0.1 μM TTR and before electrophoresis was incubated in 50 mM tris-HCL, pH 6.8 for 5 minutes at room temperature (Fig. 1, lane 1), or was boiled for 10 minutes in 50 mM tris-HCL, pH 6.8, 2% SDS and 0.2 M ß-mercaptoethanol (Fig. 1(g), lane 2).

Samples were mixed 1:1 with 2x loading buffer 100 mM tris-HC1, pH 6.8 and analyzed by 13% tris-tricine SDS-PAGE (**A**) or by 12% tris-glycine SDS-PAGE (**B, C, D, E**). The gels were dried and exposed to an X-ray X-Omat film from Kodak.

Time course experiments of complex formation demonstrated rapid formation of TTR-ßAP complexes (Figs. 1 b and c) even in the presence of ApoE3 (Fig. 1(c)).

The experiments were repeated with ^{125}I-ßAP$_{1-28}$, and the same results were obtained.

When radiolabeled ßAP$_{1-28}$ or ßAP$_{1-40}$ was added to CSF samples, instead of aggregates, two bands with apparent molecular weights of 30 and 50 kDa were observed (Fig. 1b). These bands were distinct from the 40 kDa apoE-ßAP complexes (Fig. 1b, lane 3) that were previously described (W.J. Strittmatter et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8098 (1993); J. Ghiso et al., *Biochem. J.* **293**, 27 (1993); W.J. Strittmatter et al., *Experimental Neurology* **122**, 327 (1993)). The formation of radiolabeled ßAP complexes in CSF could be specifically competed with unlabeled ßAP (Fig. 1(d), lane 3.

B.   The CSF protein that formed a 30 kDa complex with ßAP was purified, subjected to trypsin digestion, and the two largest peptides were sequenced.

- 25 -

The purification of ßAP binding activity was monitored using ^{125}I-ßAP$_{1-28}$ and SDS-PAGE and included three steps. Step 1: Chromatography of 5 ml CSF on a DEAE column and elution with a step-gradient in 50 mM tris-HCL, pH 7.4. The peak of binding activity was eluted at 0.4 M NaCl. Step 2: The peak fractions were combined, diluted five times and further passed through a heparin-sepharose column in 50 mM NaCl, 50 Mm tris-HCl pH 7.4. The ßAP binding activity appeared in unbound fractions. Step 3: The combined fractions containing ßAP binding activity were chromatographed on a FPLC-mono Q column with a gradient 0.1 - 0.3 M NaCl in 50 mM tris-HCl, pH 7.4. Fractions with peak activity from several experiments were combined, and 200 µg of purified protein were concentrated on a Speed Vac concentrator, reduced and alkylated. The protein was digested with trypsin and separated by reverse phase HPLC. The two largest peptides were sequenced by automated Edman degradation with an Applied Biosystems 477A sequencer with online PTH analysis using an Applied Biosystems 120A HPLC. The result of sequence analysis identified the sequences as **ALGISPFHEHAEVVFTANDSGP** and **RYTIAALLSPYSYSTTAVVTNPK**.

C.   The identified amino acid sequences of the two largest peptide sequences perfectly matched amino-acid residues 81 to 102 and 104 to 127, respectively, of transthyretin (TTR), a transporter of thyroxine and vitamin A in the brain (M.D. Benson and M.R. Wallace in *The Metabolic Basis of Inherited Disease*, C.R. Scriver, A.L. Beudet, W.S. Sly and D. Valle Eds. (McGraw-Hill Book Co., New York, 1989), pp. 2439-2460; D.R. Jacobson and J.N. Buxbaum in *Advances in Human Genetics* Vol. 20, H. Harris and K. Hirschhorn Eds. (Plenum Press, New York, 1991), pp. 69-123)). Commercial human plasma TTR also formed 30 kDa complexes with ßAP$_{1-28}$ that could be competed with unlabeled ßAP$_{1-40}$ demonstrating specificity of binding (Fig. 1(f), lane 3. TTR is a homotetrameric protein with

- 26 -

127 amino-acid residues in each chain, which dissociates
to form 30 kDa dimers in SDS, and 15 kDa monomers after
boiling in SDS with reducing agents (R. Murrell et al., *J.
Biol. Chem.* 267 16595 (1992)). The 30 kDa TTR-ßAP
complexes appeared as 15 kDa complexes after boiling in
SDS with reducing agents, suggesting that TTR monomer
binds ßAP (Fig. 1(g)). Using similar analytical
techniques, we identified the CSF protein that formed the
50 kDa complex with radiolabeled ßAP as albumin.

Example 2

We determined that TTR is the major ßAP binding
protein in CSF. Unlabeled ßAP was incubated with CSF, TTR
or bovine serum albumin, and the complexes were analyzed
using Western blot techniques with anti-TTR and anti-ßAP
antibodies. Ten microgram of βAP_{1-40} was incubated in 40 μl
samples overnight at 37°C in PBS, pH 7.2 with either 10 μ
CSF (Fig. 3(a), lane 1), 1 μg TTR (lane 2), or 50 μg BSA
(lane 3). Controls consisted of ten microliters CSF (lane
4) or one microgram TTR (lane 5) in 40 μl PBS, pH 7.2
without ßAP. The samples were analyzed by SDS-PAGE and
immunoblotting using rabbit anti-ßAP antibody SGY2134
kindly provided by Steven G. Younkin from Case Western
Reserve University, Cleveland, Ohio. The same results
were obtained with βAP_{1-28}.

Ten microgram βAP_{1-28} and 10 μl CSF were incubated in a
40 μl PBS, pH 7.2 overnight at 37°C. The sample was
analyzed by SDS-PAGE and immunoblotting. (Fig. 3(b)).
The membrane was cut lengthwise in two strips. One strip
was immunostained with rabbit anti-ßAP antibody SGY2134
(lane 1); the other strip was immunostained with sheep
anti-TTR antibody, ICN Biochemicals, Inc. (lane 2).
Immunoreactive proteins were detected by ECL method
(Amersham). The same results were obtained with βAP_{1-40}.

- 27 -

Only TTR-ßAP complexes with an apparent molecular weight of 30 kDa under non-reducing conditions were observed (Fig. 3). Complexes of ßAP with purified albumin or with albumin in CSF were not detected. Thus, radioiodination of ßAP by Bolton-Hunter reagent may cause the nonspecific binding of radiolabeled peptide to albumin.

Example 3

The effect of ßAP binding proteins on aggregation of unlabeled $ßAP_{1-28}$ was tested by a quantitative thioflavin-T fluorometric assay (H. LeVine III. *Protein Science* **2**, 404 (1993). TTR was purchased from Calbiochem; bovine serum albumin, fraction V, was from Sigma; human serum apoE3 and apoE4 were isolated from human plasma as described by S.C. Rall, Jr., K.H. Weisgraber, R.W. Mahley, *Methods Enzymol.*, **128**, 273 (1986)). It has been suggested that apoE may promote amyloid formation (T. Wisniewski, A. Golabek, E. Matsubara, J. Ghiso, and B. Frangione, *Biochem. Biophys. Res. Commun.* **192**, 359 (1993). Therefore, in addition to TTR, two isoforms of apoE and albumin were tested. (Fig. 4). The effect of different concentrations of bovine serum albumin (crosses), TTR (squares), ApoE3 (triangles), and apoE4 (circles) on $ßAP_{1-28}$ aggregation was determined using an thioflavin T based fluorometric assay.

One hundred percent aggregation equals the average fluorescence signal of ßAP. Synthetic $ßAP_{1-28}$ at 300 μM in water was mixed with the indicated concentrations of BSA, TTR, apoE3, or ApoE4 and aggregation was initiated with 100 mM sodium acetate, pH 5.2. After 18 hours, 5 μl samples were mixed with 10 μM Thioflavin-T in 50 mM KPO_4 and the fluorescence was measured in arbitrary units at 450 nm excitation and 482 nm emission on a Perkin-Elmer LS-50 Fluorimeter (H. LeVine III. *Protein Science* **2**, 404 (1993). TTR was purchased from Calbiochem; bovine serum

- 28 -

albumin, fraction V, was from Sigma; human serum apoE3 and
apoE4 were isolated from human plasma as described by S.C.
Rall, Jr., K.H. Weisgraber, R.W. Mahley, *Methods Enzymol.*,
128, 273 (1986)).

Transthyretin, apoE3 and apoE4 reduced the
fluorescence signal, indicating the prevention of
synthetic ßAP$_{1-28}$ aggregation, while albumin had no effect
(Fig. 4). Inhibition of ßAP$_{1-28}$ aggregation was dose
dependent with a 50% reduction in signal observed at 1.4
μM for TTR and 0.4 μM for apoE3 or apoE4.

When amyloid found in patient tissues or aggregated
synthetic ßAP is stained with congo red, it produces a
specific green to yellow birefringence when viewed under
polarized light (D.J. Selkoe, *Neuron* **6**, 487 (1991); D.L.
Price, D.R. Borchelt and S.S. Sisodia, *Proc. Natl. Acad.
Sci. U.S.A.* **90**, 6381 (1993). Congo red staining of ßAP$_{1-28}$
aggregates in the presence of 5 μM BSA (right panel) or 3
μM TTR (left panel) was therefore determined. Figure 5.
Synthetic ßAP$_{1-28}$ at 300 μM was mixed with 5 μM BSA (left
panel) or 3 μM TTR (Fig. 5, right panel) and aggregation
was initiated with 100 mM sodium acetate, pH 5.2. After
18 hours, samples were mixed with 0.2% Congo red in 100 mM
sodium acetate, pH 5.2 and 5 μl was spotted onto a
microscope slide.

We found that the addition of albumin prior to
aggregation of ßAP$_{1-28}$ did not prevent the appearance of
birefringence (Fig. 5, right panel). In contrast, when
TTR or apoE was added to ßAP$_{1-28}$ prior to aggregation, fewer
or no characteristic aggregates producing birefringence
were observed (Fig. 5, left panel).

Another feature of amyloid is the formation of
fibrils with a characteristic electron microscopic pattern
(D.J. Selkoe, *Neuron* **6**, 487 (1991); D.L. Price, D.R.
Borchelt and S.S. Sisodia, *Proc. Natl. Acad. Sci. U.S.A.*
90, 6381 (1993); E.M. Castano et al., *Biochem. Biophys.
Res. Commun.* **141**, 782 (1986); D. Burdick et al., *J. Biol.*

- 29 -

Chem. **267**, 546 (1992); J.T. Jarrett and P.T. Lansbury,
Jr., *Cell*, **73**, 1055 (1993)). Synthetic ßAP$_{1-28}$ readily
forms these typical 5-10 nm thick amyloid fibrils (Fig. 6,
right panel). ßAP$_{1-28}$ aggregates without (right panel) or
with 2 μM TTR (left panel) were analyzed using electron
microscopy. One hundred microgram of ßAP$_{1-28}$ was dissolved
in water at concentration 100 μg/ml, sonicated for 15
seconds, added to 2 μM TTR, incubated for 16 hours at 37°C
in PBS pH 7.2, and stained with 2% uranyl acetate.
Samples were examined and photographed at magnification of
25,000 on a Hitachi-12 electron microscope.

When ßAP was incubated with TTR, only amorphous
masses with few abortive short fibrils were observed,
suggesting that TTR prevented formation of characteristic
fibrils (Fig. 6, left panel). ApoE3 or apoE4 had the same
effect as TTR (data not shown).

Example 4

In order to define the binding sites of ßAP and TTR
we built three dimensional molecular models of the TTR-ßAP
complex on computers. The molecular and solvent
accessible surfaces of ßAP and TTR were generated and
electrostatic potentials were calculated using the
Poisson-Boltzmann equation. We conducted these modellings
on a Silicon Graphics Iris, 220 GTX. Coordinates for ßAP$_{1-}$
$_{28}$ correspond to the solution structure as determined by
2D-NMR and distance geometry/simulated annealing (J.
Talafous, K.J. Marcinowski, G. Klopman and M.G. Zagorski,
manuscript submitted for publication). Coordinates for
the structure of TTR had been determined by X-ray
crystallography (C.C.F. Blake, M.J. Geisow, and S.J.
Oatley, *J. Mol. Biol.* **121**, 339 (1978)). It is strikingly
clear that the electrostatic potentials of the alpha
helical ßAP$_{1-28}$ are very dipolar in nature (Fig. 7, top
panel). Likewise, TTR has clearly demarcated regions that

- 30 -

spawn negative (-) or positive (+) electrostatic
potentials (Fig. 7, center panel). The binding sites on
ßAP and TTR which would give the best fit between ßAP and
TTR were determined using the following constraints: (1)
maximize surface contacts; (2) maintain the relative
orientations to enhance electrostatic attraction; (3) bind
the amyloid peptide to each subunit of TTR independently;
and (4) avoid the TTR monomer surface involved in tetramer
formation. Using these constraints we identified the
binding scheme which is shown in Figure 7, bottom panel,
with TTR subunits (gray) and two ßAP$_{1-28}$ molecules (white).

Top (ßAP) and center (TTR dimer) panels represent
molecular surfaces shown in white and calculated
electrostatic potentials shown as surface contours. The
-1 kT potential contour is shown by the grid labelled (-)
and the +1 kT potential contour is shown by the grid
labelled (+). Bottom panel represent TTR-ßAP$_{1-28}$ complex as
a space filling model. For clarity monomers of TTR dimer
are shown in shades of gray. Two ßAP molecules are shown
in white (19).

The following amino-acid residues were found on the
contacting surface of ßAP: Arg (5), Ser (8), Gly (9), Val
(12), His (13), Gln (15), Lys (16), Phe (19), Phe (20),
Asp (23), Val (24), Asn (27), Lys (28).

In addition, the following amino-acid residues were
found on the contacting surface of TTR dimer: Arg (34,
161), Ala (37, 164), Asp (38, 165), Thr (40, 167), Glu
(42, 169), Glu (62, 189), Val (65, 192), Glu (66, 193).
The first number in parenthesis is the residue number for
the first subunit and the second number for the second
subunit of TTR.

While not wishing to be bound by theory, we believe
that the specific contribution of the amino acids on the
TTR surface which binds specifically to the ßAP peptide is
twofold. First, they provide the building blocks for the
detailed shape of the surface. Second, they provide the

- 31 -

charge that engenders a <u>positive electrostatic potential</u> which covers the <u>whole</u> <u>surface</u> with a value of +1 kT (at T =298 °K, k is the Boltzmann constant) at approximately 2 to 5 Å from the solvent accessible surface. A concave, positive potential inducing surface of $ßAP_{1-28}$ containing the residues Arg (5), His (13), Lys (16) and Lys (28) was identified which matches remarkably well with the convex negative potential inducing surface on TTR containing the residues Asp (38, 165), Glu (42, 169), Glu (62, 189), and Glu (66, 193).

Our experiments clearly show that sequestered ßAP cannot participate in amyloid fibril formation. While TTR is not the only protein that binds ßAP (W.J. Strittmatter et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8098 (1993); J. Ghiso et al., *Biochem. J.* **293**, 27 (1993); W.J. Strittmatter et al., *Experimental Neurology* **122**, 327 (1993)), it is the major ßAP sequestering protein in human CSF. The concentration of TTR is two orders of magnitude greater than concentration of ßAP and is higher than the concentration of other known ßAP binding proteins in CSF. The approximate concentrations are 3 nM for ßAP, 2 μM for albumin, 0.3 μM for TTR, 0.1 μM for apoE, 0.03 μM for apoJ, and 0.03 μM for APP (P. Seubert et al., *Nature* **359**, 325 (1992); M. Shoji et al., *Science* **258**, 126 (1992); B.A. Yankner, L.K. Duffy and D.A. Kirschner, *Science* **250**, 279 (1990); D.M. Araujo and C.W. Cotman, *Brain Res.* **569**, 141 (1992); M.P. Matson, et al., *J. Neurosci.* **12**, 376 (1992); C. Behl, J. Davis, G.M. Cole and D. Schubert, *Biochem. Biophys. Res. Commun.* **186**, 944 (1992)). Our data do not exclude the possibility that other proteins form complexes with ßAP; however, most, if not all, ßAP is probably sequestered by TTR (Figs. 1 and 3).

- 32 -

Example 5

Identification of TTR mutation.

Genetic linkage studies have reported several loci for familial AD (FAD) on chromosomes 21,14,19. However many FAD pedigrees have not shown evidence for linkage to these chromosomes, suggesting a genetically heterogeneous mechanism of disorder and existence of additional FAD susceptible genes.

Because of TTR's importance in amyloid formation, the TTR gene could be a candidate for FAD and sporadic AD in families not linked to defined genetic loci on chromosomes 14,19 and 21 or modulate effect of defined loci.

In order to identify a mutation in the TTR gene associated with AD, we have analyzed by PCR-Single Strand Conformation Polymorphism (SSCP), the sequence of three TTR exons in 55 unrelated AD patients.

PCR-SSCP analysis was performed according to the method of Orita et al. (Orita M, Suzuki Y, Sekiya T., Hayashi K., Genomics 5: 874-879). In all cases, one 5' end-labeled primer and one unlabeled primer were used for genomic PCR amplification. Oligonucleotides for all exons are listed in Table 1.

TABLE 1

Exon	Oligonucleotide	Position
2	5'CGC TCC AGA TTT CTA ATA CCAC 3'	1515-1537
	5'AGT GAG GGG CAA ACG GGA AGAT 3'	1791-1769
3	5'TGG TGG GGG TGT ATT ACT TTGC 3'	3446-3468
	5'CAT TTC CTG GAA TGC CAA AAGC 3'	4022-4000
4	5'GGT CAG TCA TGT TGT TCA TCTG 3'	7193-7215
	5'TAG TAA AAA TGG AAT ACT CTTG 3'	7447-7425

- 33 -

Primers were 5' end labeled with 32p-ATP using T4
polynucleotide kinase according to manufacturers
instructions (New England BioLabs). The PCR mixture
contained 12.5 ng of both primers, 5nmol of dNTP, 250 ng
of genomic DNA and 1.25 U of AmpliTag polymerase (Perkin-
Elmer Cetus). PCR was performed in a Perkin ElmerCetus
DNA thermal cycler for 30 cycles (each cycle was 94 C-
1min, 56 C-1 min, 72 C -2min, Extension 72-10 min.
Electrophoresis was carried out at 4 C in 6%
polyacrylamide gel.

PCR-SCCP revealed a polymorphism in exon 2 in 7
patients with AD. The amplified sequence of exon 2 was
cloned in the pCR™ vector (Invitrogen) and target DNA was
sequenced using a DNA Sequencing KIT (USB).

Ten (10) patients had a heterozygous G-A substitution
in codon 6, which changed the normal glycine codon, GGT,
to one for serin, AGT. This TTR variant originally was
described as a variant with elevated thyroxine-binding
affinity (Fitch N.J.S. et al.,Journal of Endocrinology,
1991, 129, 309-313.). Although the gene frequency of the
serine 6 TTR variant was found to be 12% in North American
Caucasians (Jacobsen et al. supra.), we have found that
the frequency of this variant in our random selected
population of AD patients increased up to 18%.

G-A substitution in serine 6 variant of TTR creates a
BsrI site. This enzyme might therefore be used for RFLP
analysis of the TTR gene in AD pedigrees. Using the same
conditions and the same primers for exon 2 (Table 1), we
analyzed the PCR product for the presence of the BsrI
restriction site (Fig. 8). These data show that the
serine 6 TTR variant, as well as other TTR variants, could
be associated with AD and can provide a genetic diagnostic
test for some forms of AD.

While we have hereinbefore described a number of
embodiments of this invention, it is apparent that the
basic constructions can be altered to provide other

- 34 -

embodiments which utilize the methods of this invention. Therefore, it will be appreciated that the scope of this invention is defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

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- 35 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i)   THE RESEARCH FOUNDATION OF STATE UNIVERSITY
            OF NEW YORK

   (ii)   TITLE OF INVENTION: METHOD OF PREVENTING
            AGGREGATION OF AMYLOID β-PROTEIN

   (iii)   NUMBER OF SEQUENCES: 8

   (iv)   CORRESPONDENCE ADDRESS:
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            (D)   STATE: NEW YORK
            (E)   COUNTRY: USA
            (F)   ZIP: 10154

   (v)   COMPUTER READABLE FORM:
            (A)   MEDIUM TYPE: FLOPPY DISK
            (B)   COMPUTER: IBM PC COMPATIBLE
            (C)   OPERATING SYSTEM: PC-DOS/MS-DOS
            (D)   SOFTWARE: WORDPERFECT 5.1

   (vi)   CURRENT APPLICATION DATA:
            (A)   APPLICATION NUMBER:
            (B)   FILING DATE: 03-NOV-1993
            (C)   CLASSIFICATION:

   (vii)   PRIOR APPLICATION DATA:
            (A)   APPLICATION NUMBER: US08/148,117
            (B)   FILING DATE: 04-NOV-1993
            (C)   CLASSIFICATION:

   (viii)   ATTORNEY/AGENT INFORMATION:
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            (B)   REGISTRATION NUMBER: 33,285
            (C)   REFERENCE/DOCKET NUMBER: 0887-4113

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            (B)   TELEFAX: (212) 751-6849
            (C)   TELEX: 421792

(2) INFORMATION FOR SEQ ID NO:1:

   (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 22

- 36 -

```
              (B)  TYPE:  NUCLEOTIDE
              (C)  STRANDEDNESS:  SINGLE
              (D)  TOPOLOGY:  UNKNOWN


      (xi)     SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCTCCAGAT TTCTAATACC AC                                    22


  (2)   INFORMATION FOR SEQ ID NO:2:

      (i)      SEQUENCE CHARACTERISTICS:
              (A)  LENGTH:  22
              (B)  TYPE:  NUCLEOTIDE
              (C)  STRANDEDNESS:  SINGLE
              (D)  TOPOLOGY:  UNKNOWN


      (xi)     SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGTGAGGGGC AAACGGGAAG AT                                    22


  (2)   INFORMATION FOR SEQ ID NO:3:

      (i)      SEQUENCE CHARACTERISTICS:
              (A)  LENGTH:  22
              (B)  TYPE:  NUCLEOTIDE
              (C)  STRANDEDNESS:  SINGLE
              (D)  TOPOLOGY:  UNKNOWN


      (xi)     SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGTGGGGGT GTATTACTTT GC                                    22


  (2)   INFORMATION FOR SEQ ID NO:4:

      (i)      SEQUENCE CHARACTERISTICS:
              (A)  LENGTH:  22
              (B)  TYPE:  NUCLEOTIDE
              (C)  STRANDEDNESS:  SINGLE
              (D)  TOPOLOGY:  UNKNOWN


      (xi)     SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATTTCCTGG AATGCCAAAA GC                                    22
```

- 37 -

(2)   INFORMATION FOR SEQ ID NO:5:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 22
        (B)   TYPE: NUCLEOTIDE
        (C)   STRANDEDNESS: SINGLE
        (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTCAGTCAT GTTGTTCATC TG   22

(2)   INFORMATION FOR SEQ ID NO:6:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 22
        (B)   TYPE: NUCLEOTIDE
        (C)   STRANDEDNESS: SINGLE
        (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:6:

TAGTAAAAAT GGAATACTCT TG   22

(2)   INFORMATION FOR SEQ ID NO:7:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 22
        (B)   TYPE: AMINO ACID
        (C)   STRANDEDNESS: SINGLE
        (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Leu Gly Ile Ser Pro Phe His Glu His Ala Glu
1   5   10

Val Val Phe Thr Ala Asn Asp Ser Gly Pro
        15   20

(2)   INFORMATION FOR SEQ ID NO:8:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 23
        (B)   TYPE: AMINO ACID
        (C)   STRANDEDNESS: SINGLE
        (D)   TOPOLOGY: UNKNOWN

- 38 -

(xi)　　　SEQUENCE DESCRIPTION: SEQ ID NO:8:

Arg Tyr Thr Ile Ala Ala Leu Leu Ser Pro Tyr Ser
1   5   10

Tyr Ser Thr Thr Ala Val Val Thr Asn Pro Lys
        15   20

- 39 -

WE CLAIM:

1.   A method of preventing aggregation of free ßAP comprising, providing a ßAP-binding compound (BBC) to ßAP present in a solution wherein the BBC is provided in an amount sufficient to form a BBC-ßAP complex and wherein the BBC is capable of preventing amyloid formation by the ßAP.

2.   The method of preventing aggregation of ßAP according to claim 1, wherein the BBC comprises a binding domain which binds to the surface of the ßAP at a site defined by the amino acid residues Arg (5), Ser (8), Gly (9), Val (12), His (13), Gln (15), Lys (16), Phe (19), Phe (20), Asp (23), Val (24), Asn (27), and Lys (28), and wherein the BBC is provided to the solution in an amount sufficient to decrease the concentration of free amyloid ß-protein in the solution.

3.   The method according to claim 2 wherein the BBC comprises a binding site which binds to amino acid residues Arg (5), His (13), Lys (16), and Lys (28) of ßAP.

4.   The method according to claim 3 wherein the BBC is selected from the group consisting of TTR and apoE.

5.   The method according to claim 5 wherein the complexes are stable in SDS.

6.   The method according to claim 5 wherein the BBC is TTR.

7.   The method according to claim 1 wherein the BBC is provided to the solution so as to achieve a ratio of BBC to soluble ßAP of between about 1:100 to about 100:1.

- 40 -

8.   The method according to claim 7 wherein the ratio is between about 1 to about 4.

9.   The method according to claim 8 wherein the ratio is about 1 to about 2.

10.   The method according to claim 8 wherein the BBC is TTR.

11.   A method of determining the amount of soluble amyloid ß-protein (ßAP) present in a sample comprising the steps of
        combining a sample comprising ßAP with a BBC according to claim 1 and a known amount of soluble ßAP, wherein the BBC is either soluble or bound to a solid support, and detecting the amount of ßAP in the sample.

12.   The method according to claim 11 wherein the BBC is TTR bound to a solid support and the known amount of soluble ßAP is labelled to allow detection.

13.   A composition for preventing the aggregation of ßAP in a sample comprising the BBC according to claim 1 and a physiologically inert carrier.

14.   The composition according to claim 13 wherein the BBC is TTR.

15.   A method of reducing the amount of soluble ßAP at a given concentration *in vivo* comprising, providing a BBC according to claim 1 to a mammal in an amount sufficient to bind to the soluble ßAP so as to reduce the concentration of free ßAP.

16.   The method according to claim 15 wherein the BBC is TTR.

- 41 -

17.   The method according to claim 16 wherein TTR is provided to the mammal by inducing endogenous production of TTR.

18.   A method for detecting a person at risk for developing ßAP associated amyloidosis comprising, obtaining a sample of DNA from a person and analyzing the DNA for the presence of a mutation in the TTR gene characterized by a substitution of serine for glycine at amino acid residue number 6.

19.   The method according to claims 18 wherein the person is at risk for Alzheimer's Disease.

20.   A compound capable of binding to ßAP to prevent ßAP aggregation wherein the compound comprises a binding domain which binds to the surface of the ßAP at a site defined by the amino acid residues Arg (5), Ser (8), Gly (9), Val (12), His (13), Gln (15), Lys (16), Phe (19), Phe (20), Asp (23), Val (24), Asn (27), and Lys (28), with the proviso that the BBC is not TTR.

21.   The compound according to Claim 20 wherein the compound comprises a binding site which binds to amino acid residues Arg (5), His (13), Lys (16), and Lys (28) of amyloid ß-protein.

22.   The compound according to claim 21 wherein the compound is a polypeptide.

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

Lane 1 2 3 4 5 6 7

FIG. 8

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6)   :G01N 33/566; C07K 3/00
US CL   :436/501; 530/350+
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   436/501; 530/350+

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,164,295 (KISILEVSKY ET AL) 17 NOVEMBER 1992, see entire document.	1-14 and 18-22
X	EMBO Journal, Volume 12, No. 2, issued 1993, C. Thylen et al, "Modifications of Transthyretin in Amyloid Fibrils: Analysis of Amyloid from Homozygous and Heterozygous Individuals with the Met30 Mutation", 743-748, see entire document.	1-14 and 18-22
X	Scandinavian Journal of Immunology, Volume 38, issued 1993, P.M.P. Costa, "Immunoassay for Transthyretin Variants Associated with Amyloid Neuropathy", pages 177-182, see entire document.	1-14 and 18-22

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document published on or after the international filing date	
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 FEBRUARY 1995	17 FEB 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DEBORAH CROUCH
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,137,873 (YANKNER) 11 August 1992, see entire document.	15-19

INTERNATIONAL SEARCH REPORT

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.
Group I, claims 1-14 and 20-22, drawn to an in vitro method to prevent aggregation of βAP in a sample, a composition and a compound, classified respectively in Class 435, subclass 7.1 and Class 530, subclass 350+.
Group II, claims 15-17, drawn to an in vivo method to prevent aggregation of βAP, classified in Class 514, subclass 2.
Group III, claims 18 and 19, drawn to a method of assaying DNA to determine if a person is at risk for Alzheimer's Disease, classified in Class 435, subclass 6.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The invention of group I is drawn to an in vitro method of assay to determine the amount βAP present in a sample and a method to prevent aggregation of βAP in the sample, along with compositions for said preventative step. The invention of group II is to a method of treating an individual. The invention of group III is to a method of assay by DNA analysis. Thus the in vitro methods require separate protocols for administration than the in vivo method. The in vitro methods are materially different as in group I, a protein is being measured and group II, DNA sequence is being analyzed. The methods are not required for each other. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

(51) International Patent Classification 6 : C07K 14/705, 14/34, 14/235, 14/445, 14/165, 14/77, 14/12, 14/135, 14/11, 14/62, 14/02, 14/09, 14/145, 16/28, 16/08, 16 /10, 16 /12, 16 /18, 16 /20, 16 /26, A61K 39 /00, 39 /015, 39 /05, 39 /10, 39 /145, 39 /135, 39 /165, 39 /205, 39 /29, 39 /39	A1	(11) International Publication Number: **WO 95/23166** (43) International Publication Date: 31 August 1995 (31.08.95)

(21) International Application Number: PCT/AU95/00090 (22) International Filing Date: 24 February 1995 (24.02.95) (30) Priority Data: PM 4119 25 February 1994 (25.02.94) AU (71) Applicant *(for all designated States except US)*: DEAKIN RESEARCH LIMITED [AU/AU]; Level 1, 80 Mount Street, North Sydney, NSW 2060 (AU). (72) Inventors; and (75) Inventors/Applicants *(for US only)*: COMIS, Alfio [AU/AU]; 3 Prairie Vale Road, Bossley Park, NSW 2176 (AU). TYLER, Margaret, Isabel [AU/AU]; 43A Trentino Road, Turramurra, NSW 2074 (AU). FISCHER, Peter [CH/NO]; Nycomed Bioreg A/S, Gaustadalleen 21, N-0371 Oslo (NO). (74) Agent: GRIFFITH HACK & CO.; G.P.O. Box 4164, Sydney, NSW 2001 (AU).	(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). **Published** *With international search report.*

(54) Title: SYNTHETIC INVERSO OR RETRO-INVERSO T-CELL EPITOPES

(57) Abstract

Synthetic T cell epitope analogues of native T cell epitopes which are partially or completely inverso or retro-inverso modified with respect to the native T cell epitope are shown to be effective as T cell epitopes. These T cell epitope analogues stimulate immune responsiveness when used in place of their native T cell epitope counterparts in vaccines. The invention further relates to the use of these T cell epitope analogues, to vaccines comprising the T cell epitope analogues, to methods of preparing vaccines comprising these T cell epitope analogues, and to antibodies generated using these T cell epitope analogues.

SYNTHETIC INVERSO OR RETRO-INVERSO T-CELL EPITOPES

TECHNICAL FIELD

The present invention relates to synthetic T cell
epitope analogues of native T cell epitopes with partial

5   or complete inverso or retro-inverso modifications.
These T cell epitope analogues stimulate immune
responsiveness when used in place of their native T cell
epitope counterparts in vaccines. The invention further
relates to the use of these T cell epitope analogues, to

10   vaccines comprising the T cell epitope analogues, to
methods of preparing vaccines comprising these T cell
epitope analogues, and to antibodies generated using
these T cell epitope analogues.

BACKGROUND ART

15   The stereochemistry of polypeptides can be described
in terms of the topochemical arrangement of the side
chains of the amino acid residues about the polypeptide
backbone which is defined by the peptide bonds between
the amino acid residues and the α-carbon atoms of the

20   bonded residues. In addition, polypeptide backbones have
distinct termini and thus direction.
The majority of naturally occurring amino acids are
L-amino acids. Naturally occurring polypeptides are
largely comprised of L-amino acids.

25   D-amino acids are the enantiomers of L-amino acids
and form peptides which are herein referred to as inverso
peptides, that is, peptides corresponding to native
peptides but made up of D-amino acids rather than L-amino
acids.

30   Retro-inverso modification of naturally occurring
polypeptides involves the synthetic assemblage of amino
acids with α-carbon stereochemistry opposite to that of
the corresponding L-amino acids, i.e. D- or D-allo-amino
acids, in reverse order with respect to the native

35   peptide sequence. A retro-inverso analogue thus has
reversed termini and reversed direction of peptide bonds

while approximately maintaining the topology of the side
chains as in the native peptide sequence.

Partial retro-inverso peptide analogues are
polypeptides in which only part of the sequence is
reversed and replaced with enantiomeric amino acid
residues. Since the retro-inverted portion of such an
analogue has reversed amino and carboxyl termini, the
amino acid residues flanking the retro-inverted portion
are replaced by side-chain-analogous α-substituted
geminal-diaminomethanes and malonates, respectively.

Processes for synthesis of retro-inverso peptide
analogues (Bonelli et al., 1984; Verdini and Viscomi,
1985) and some processes for the solid-phase synthesis of
partial retro-inverso peptide analogues have been
described (Pessi et al., 1987).

It has been observed that due to the
stereospecificity of enzymes with respect to their
substrates, replacement of L-amino acid residues with
D-amino acid residues in peptide substrates generally
abolishes proteolytic enzyme recognition and/or activity,
although exceptions are known.

Peptide hormones have been of particular interest as
targets for retro-inversion, presumably because their
analogues would have potential use as therapeutic agents.
Partial, and in a few cases complete, retro-inverso
analogues of a number of peptide hormones have been
prepared and tested (see, for example, Goodman and
Chorev, 1981).

Complete or extended partial retro-inverso analogues
have generally been found to be devoid of biological
activity. The lack of biological activity has been
attributed to possible complex structural changes caused
by extended modification, the presence of reversed chain
termini or the presence of proline residues in the
sequences. Some partial retro-inverso analogues, that is
peptides in which only selected residues were modified,
on the other hand, have been shown to retain or enhance
biological activity. Retro-inversion has also found

application in the area of rational design of enzyme
inhibitors.

The fact that retro-inversion of biologically active
peptides has met with only limited success in retaining
or enhancing the activity of the native peptide is
probably due to several reasons. Although structurally
very similar, it was realised early that peptides and
their retro-enantiomers are topologically not identical
and crystal structure and solution conformation studies
have borne this out. Biological activity of a peptide
hormone or neurotransmitter depends primarily on its
dynamic interaction with a receptor, as well as on
transduction processes of the peptide-receptor complex.
It is now clear that such interactions are complex
processes involving multiple conformational and
topological properties. Consequently it is not
surprising that a retro-inverso analogue may not be able
to mimic all of these properties.

In order to activate the cellular component of the
immune system a vaccine must present T-cell epitopes, as
well as pathogen-specific B-cell epitopes. T cells fail
to recognise soluble antigen. They require its
presentation on the surface of antigen presenting cells
(APC) in association with molecules encoded by the major
histocompatibility complex (MHC). In the case of large
proteins which constitute conventional vaccines, the
protein undergoes enzymatic digestion intracellularly.
Some of the resulting peptide fragments can bind to MHC
molecules and the peptide-MHC complexes are then
transported to the surface of APCs. The peptides capable
of binding MHC molecules are T-cell epitopes. Because of
the genetic restriction of the MHC, the sequences which
can act as T-cell epitopes may vary amongst individuals
in an outbred population. Totally synthetic vaccines
(Jolivet et al., 1990) therefore need to be designed with
regard to these facts. While it is possible to provide
T-cell epitopes in a peptide vaccine by conjugation of
the relevant B-cell epitope peptides to a carrier protein

such as tetanus toxoid, this is not desirable because it
negates the inherent advantages of a peptide vaccine,
e.g. chemical stability and ease of production. The
identification of appropriate T-cell epitope 'cocktails'
potentially useful in synthetic vaccines is therefore an
active field of research (Schwartz, 1986).

DISCLOSURE OF THE INVENTION

Definitions

Throughout the specification and claims "retro
modified" refers to a peptide which is made up of L-amino
acids in which the amino acid residues are assembled in
opposite direction to the native peptide with respect to
which it is retro modified.

Throughout the specification and claims "inverso
modified" refers to a peptide which is made up of D-amino
acids in which the amino acid residues are assembled in
the same direction as the native peptide with respect to
which it is inverso modified.

Throughout the specification and claims "retro-
inverso modified" refers to a peptide which is made up of
D-amino acids in which the amino acid residues are
assembled in the opposite direction to the native peptide
with respect to which it is retro-inverso modified.

Throughout the specification and claims the term
"native" refers to any sequence of L amino acids used as
a starting sequence for the preparation of partial or
complete retro, inverso or retro-inverso analogues.

The term "peptide" as used throughout the
specification and claims is to be understood to include
peptides of any length.

Throughout the specification and claims the term
"antigenic fragment" refers to a peptide which is a
portion of an antigen which itself is immunogenic or
capable of binding antibodies.

The term "antigen" as used throughout the
specification and claims is to be understood to include
immunogens as the context requires.

- 5 -

Throughout the specification and claims the term "antigen analogue" refers to a peptide molecule capable of mimicking the immunological activity of the native peptide antigen with respect to which it is partially or
5   completely retro, inverso or retro-inverso modified. Retro peptides are made up of L-amino acids and are peptides in which the amino acid residues are assembled in opposite direction to the native peptide sequence.

Throughout the specification and claims the term "T-
10   cell epitope analogue" refers to a peptide molecule capable of mimicking the immunological activity of the native T-cell epitope with respect to which it is partially or completely inverso or retro-inverso modified.

15   Partial modification includes analogues in which as few as two consecutive residues are modified. Typically at least 5 or 6 consecutive residues are modified.

The present invention relates to partially or completely inverso or retro-inverso modified T-cell
20   epitope analogues of native T cell epitopes which stimulate immune responsiveness when used in place of their native T cell epitope counterparts in vaccines. Incorporation of D-amino acids into T-cell epitope analogues increases their stability to degradation after
25   administration. Further, incorporation of D-amino acids has potential for oral administration of analogues.

Having shown that particular retro-inverso or inverso T-cell epitope analogues can stimulate immune responsiveness when used in the place of their native T-
30   cell epitope counterparts it follows that, generally, these analogues can be expected to be successful since T-cell epitope - MHC molecule interactions are not fundamentally different from case to case.

In a first aspect the invention provides a synthetic
35   peptide T cell epitope analogue of a native T cell epitope, which analogue is partially or completely inverso or retro-inverso modified with respect to the native T cell epitope.

The T cell epitope analogues of the present
invention stimulate immune responsiveness when used in
place of their native T cell epitope counterparts in
vaccines.

The efficacy of T cell epitope analogues of the
invention is illustrated with respect to the malaria T
cell epitopes of Example 2.

In a second aspect the invention provides a vaccine
comprising a T cell epitope analogue of the first aspect
together with a B cell epitope and a pharmaceutically or
veterinarally acceptable carrier, diluent, excipient
and/or adjuvant. Typically, the vaccines of the
invention are cocktails of T cell epitope analogues and B
cell epitopes tailored to the condition against which
vaccination is required. Preferably the T cell epitope
analogue is conjugated to the B cell epitope.

The B cell epitope is conjugated to the T cell
epitope by standard chemical conjugation techniques or
the conjugate is synthesized as a continuous peptide.

The B cell epitope can be provided as any epitope,
or any intact molecule providing the epitope, against
which an antibody response is required.

The B cell epitopes to be incorporated into vaccines
in accordance with the invention include peptides or
polypeptides of any length whose amino acid sequences
stem from polypeptides of pathogens such as
poliomyelitis, hepatitis B, foot and mouth disease of
livestock, tetanus, pertussis, HIV, cholera, malaria,
influenza, rabies or diphtheria causing agents, or toxins
such as robustoxin, heat labile toxin of pathogenic
Escherichia coli strains and Shiga toxin from *Shigella
dysenteriae*. Other B cell epitopes of interest include
epitopes of Amyloid ß protein (Alzheimer's disease) and
human chorionic gonadotropin and gonadotropin releasing
hormone (contraceptive vaccines).

The B cell epitope is preferably a retro, retro-
inverso or inverso antigen analogue.

Preferred T cell epitope analogues of the invention

are analogues of:
Diphtheria toxin:
H-Gln-Val-Val-His-Asn-Ser-Tyr-Asn-Arg-Pro-Ala-Tyr-Ser-
Pro-Gly-OH (SEQ ID NO: 1)
5   Pertussis toxin:
H-His-Arg-Met-Gln-Glu-Ala-Val-Glu-Ala-Glu-Arg-Ala-Gly-
Arg-OH (SEQ ID NO: 2)
Malaria CSA protein:
H-Pro-Ser-Asp-Lys-His-Ile-Glu-Gln-Tyr-Leu-Lys-Lys-Ile-
10   Lys-Asn-Ser-Ile-Ser-OH (SEQ ID NO: 3)
Malaria CSB protein:
H-His-Ile-Glu-Gln-Tyr-Leu-Lys-Lys-Ile-Lys-Asn-Ser-Ile-
Ser-OH (SEQ ID NO: 4)
Malaria CST3 protein:
15   H-Gly-Asp-Ile-Glu-Lys-Lys-Ile-Ala-Lys-Met-Glu-Lys-Ala-
Ser-Ser-Val-Phe-Asn-Val-Val-Asn-Ser-OH (SEQ ID NO: 5)
Hen egg lysozyme:
H-Cys-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-
Asn-Cys-Ala-OH (SEQ ID NO:6)
20   Ovalbumin:
H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-
Glu-OH (SEQ ID NO: 7) and
H-Tyr-Thr-Tyr-Thr-Val-His-Ala-Ala-His-Ala-Tyr-Thr-Tyr-
Thr-OH (SEQ ID NO: 8)
25   Other preferred T cell epitope analogues are
analogues of:
Measles Virus F and H glycoproteins: (Partidos C.D. et
al, 1991)
MVF:258-277   H-Gly-Ile-Leu-Glu-Ser-Arg-Gly-Ile-Lys-Ala-
30   Arg-Ile-Thr-His-Val-Asp-Thr-Glu-Ser-Tyr-OH
                 (SEQ ID NO: 9)
MVF:288-302   H-Leu-Ser-Glu-Ile-Lys-Gly-Val-Ile-Val-His-
                 Arg-Leu-Glu-Gly-Val-OH (SEQ ID NO: 10)
Respiratory syncytial virus 1A protein: (Nicholas J.A. et
35   al, 1988)
RS1A:45-60   H-Cys-Glu-Tyr-Asn-Val-Phe-His-Asn-Lys-Thr-
                 Phe-Glu-Leu-Pro-Arg-Ala-OH (SEQ ID NO: 11)
Influenza hamagglutinin A/PR/8/34 Mt.S.:

109-119	(Hackett C.J. *et al* 1983)	(SEQ ID NO: 12)
130-140	(Hurwitz J.J. *et al* 1984)	(SEQ ID NO: 13)
302-313	(Lamb J.R. *et al* 1982; Hurwitz J.L. *et al* 1984) (SEQ ID NO: 14)	

5   Pork Insulin:

(A)4-14   (Rosenthal A.S. 1978) (SEQ ID NO: 15)

(B)5-16   (Thomas J.W. *et al* 1981) (SEQ ID NO: 16)

Hepatitis B virus pre S:

120-132   (Milich D.R. *et al* 1986) (SEQ ID NO: 17)

10 Hepatitis B virus major surface antigen:

38-52   (Milich D.R. *et al* 1985) (SEQ ID NO: 18)

95-109   "   (SEQ ID NO: 19)

140-154   "   (SEQ ID NO: 20)

Foot and mouth virus VP1:

15 141-160   (Francis M.J. *et al* 1985) (SEQ ID NO: 21)

Rabies virus-spike glycoprotein precursor:

32-44   (Macfarlan R.I. *et al* 1984) (SEQ ID NO: 22)

In a third aspect the invention provides a method of

20 vaccinating a host in need of such treatment which method
comprises administering an effective amount of a vaccine
according to the second aspect to the host.

In a fourth aspect the invention provides antibodies
produced by immunisation of a host with a vaccine of the

25 second aspect.

In a fifth aspect the invention provides a method of
preparing a T cell epitope analogue of the invention
comprising synthesising a partially or completely inverso
or retro-inverso peptide comprising the analogue.

30   In a sixth aspect the invention provides a method of
preparing a vaccine of the second aspect comprising
conjugating a T cell epitope analogue of the first aspect
to a B cell epitope or admixing a T cell epitope analogue
of the first aspect with a B cell epitope and admixing an

35 effective amount of the resulting mixture or conjugate
with a pharmaceutically or veterinarally acceptable
carrier, diluent, excipient and/or adjuvant.

Vaccines of the invention can be formulated using

- 9 -

standard methods in the art of vaccine formulation.
        Selection of appropriate diluents, carriers,
excipients and/or adjuvants can be made in accordance
with standard techniques in the art.
        Vaccines of the invention may be administered to
hosts in need of such treatment by injection. Vaccines
incorporating D-amino acid containing analogues may also
be administered orally.

ABBREVIATIONS
BOP   (benzotriazolyloxy)tris(dimethylamino)
            phosphonium hexafluorophosphate (Castro's
            reagent)
DMF   dimethyl formamide
ELISA   enzyme-linked immunosorbent assay
Fmoc   9-fluorenylmethoxycarbonyl
HPLC   high-performance liquid chromatography
Ig   immunoglobulin
in   inverso
i.p.   intraperitoneal
no   normal (native)
PBS   phosphate buffered saline (10 mM phosphate,
            150mM NaCl, pH 7.4)
Pfp   pentafluorophenyl
PVC   polyvinylchloride
ri   retro-inverso
TFA   trifluoroacetic acid

Amino Acids:
        L-amino acids are indicated by an upper case
followed by lower case lettering e.g. Ala indicates
L-alanine.
        D-amino acids are indicated by all lower case
abbreviations, e.g. ala indicates D-alanine.

                    BRIEF DESCRIPTION OF THE FIGURES
        Figure 1 shows the results of a cell proliferation
experiment conducted using the T-cell epitope peptides

noMalCST3 (SEQ ID NO: 5), inMalCST3 and riMalCST3.

Figure 2 shows antibody production measured in mice immunized with the B-cell epitope H-(Asn-Ala-Asn-Pro)$_3$-OH (SEQ ID NO: 23) alone or together with either no or riMalCST3.

Figure 3 shows antibody production measured in mice immunized with the B-cell epitope H-(Asn-Ala-Asn-Pro)$_3$-OH (SEQ ID NO: 23) alone or together with either no (SEQ ID NO: 3) or riMalCSA protein.

Figure 4 shows antibody production measured in mice immunized with the B-cell epitope H-(Asn-Ala-Asn-Pro)$_3$-OH (SEQ ID NO: 23) alone or together with either no (SEQ ID NO: 4) or riMalCSB protein.

Figure 5 shows antibody production measured in mice immunized with the B-cell epitope H-(Asn-Ala-Asn-Pro)$_3$-OH (SEQ ID NO: 23) alone or together with either no (SEQ ID NO: 1) or riDiphT.

Figure 6 shows antibody production measured in mice immunized with the B-cell epitope H-(Asn-Ala-Asn-Pro)$_3$-OH (SEQ ID NO: 23) alone or together with either no (SEQ ID NO: 2) or riPertT.

Figure 7 shows antibody production measured in mice immunized with the B-cell epitope H-(Asn-Ala-Asn-Pro)$_3$-OH (SEQ ID NO: 23) alone or together with either no (SEQ ID NO: 7) or riOvalT.

BEST MODE OF CARRYING OUT THE INVENTION

T cell epitope analogues of the invention are prepared by standard techniques for the preparation of L and D amino acid containing peptides, particularly as outlined in Example 1.

Vaccines of the invention are formulated by standard techniques for vaccine formulation using standard carriers, diluents excipients and/or adjuvants suitable for the formulation of oral or injectable vaccines. Effective amounts of Tcell-epitope analogues to be incorporated in the vaccines can be determined in accordance with standard methods. Conjugation techniques

where used are standard chemical conjugation techniques.

The vaccination regimes used are standard regimes for the vaccination of animal or human hosts. These regimes can be used where immunisation of the host is desired or where the host is being used to produce antibodies for exogenous use.

The invention is further described in the following examples which are illustrative of the invention but in no way limiting on its scope.

10   EXAMPLE 1

Peptide Synthesis

Peptides were synthesised by a solid-phase method on polyamide (Arshady et al., 1981) or Polyhipe supports using side-chain protected Fmoc amino acids (Carpino & Han, 1972), essentially as described by Eberle et al. (1986). Only pure amino acid derivatives, obtained commercially or by synthesis, were used. The polyamide synthesis resins, derivatised with p-alkoxybenzyl alcohol-based linkage agents, were esterified quantitatively with the appropriate preformed C-terminal Fmoc-amino acid symmetrical anhydrides, in the presence of 0.2 molar equivalents of N,N-dimethylaminopyridine and N-methylmorpholine. The Polyhipe resin, derivatised with Fmoc-Rink linker (Rink, 1987) did not require esterification of the first amino acid linked to it. Chain elongation was carried out using Fmoc-amino acid pentafluorophenyl esters (Atherton et al., 1988) or Castro's reagent/1-hydroxybenzotriazole coupling (Hudson, 1988). The progress of each synthesis was monitored using a specific colour test (Hancock & Battersby, 1976) and/or amino acid analysis of acid-hydrolysed peptidyl resin samples.

The peptides were cleaved from the resins and side-chain deprotected with the aid of TFA, containing a suitable mixture of scavenger chemicals (Tam, 1988). After filtration and vacuum evaporation, the peptides were triturated with diethyl ether, collected by

centrifugation and lyophilised from aqueous ammonium
bicarbonate solution.

All peptides then underwent an initial desalting and
purification step by column chromatography on suitable
gel filtration media in aqueous solvents. Afterwards
they were purified to homogeneity by reversed-phase HPLC
using water-acetonitrile (containing 0.05-0.1% TFA)
gradient elution. The purity of the synthetic peptides
was further assessed by gas-phase acid hydrolysis/amino
acid analysis (Bidlingmeyer *et al.*, 1987) and, if deemed
necessary, by automated gas-phase sequencing (Hunkapiller
& Hood, 1983).

EXAMPLE 2
Malaria T-cell epitope peptides

It has been shown that nonresponsiveness to the
malaria immunodominant B-cell epitope (Asn-Ala-Asn-Pro)$_x$
(SEQ ID NO: 23) of the *Plasmodium falciparum*
circumsporozoite protein can be overcome in the presence
of a particular T-cell epitope peptide from the same
protein (Sinigaglia *et al*, 1988). The peptide in
question, unlike most T-cell epitopes, is recognised in
association with most human MHC class II molecules and
has been suggested as a suitable component of a synthetic
peptide vaccine against malaria. The region of the
circumsporozoite protein from which the peptide stems is
apparently conserved in different parasite isolates.

The following peptides were prepared according to
the usual protocols:

noMalCST3   H-Gly-Asp-Ile-Glu-Lys-Lys-Ile-Ala-Lys-Met-
             Glu-Lys-Ala-Ser-Ser-Val-Phe-Asn-Val-Val-Asn-
             Ser-OH (SEQ ID NO: 5)

inMalCST3   H-Gly-asp-ile-glu-lys-lys-ile-ala-lys-met-
             glu-lys-ala-ser-ser-val-phe-asn-val-val-asn-
             ser-OH

riMalCST3   H-ser-asn-val-val-asn-phe-val-ser-ser-ala-
             lys-glu-met-lys-ala-aile-lys-lys-glu-aile-
             asp-Gly-OH

BALB/c mice were immunised subcutaneously at the
base of the tail with the above T-cell epitope peptides
emulsified in an equal volume of complete Freund's
adjuvant. Ten days later, the animals were killed by
5   cervical dislocation and the inguinal and popliteal lymph
nodes removed. A cell suspension from the lymph nodes
was prepared and the cells cultured in the presence of
various concentrations of the test antigen, as well as a
non-related control antigen. Cell proliferation was
10   quantitated by measuring the incorporation into the cells
of radiolabelled thymidine. Results from the experiment
are shown in Fig. 1.

When animals were primed with any form of the
peptide and the animals' cultured T cells challenged with
15   the same peptide, proliferation was observed in every
case. Upon priming with one form of a peptide and
challenging with either of the other two forms, some
activation was observed in each case.

In order to remove any potential effects due to
20   non-specific cell proliferation, the T cell assay method
was improved as follows:

A cell suspension from the lymph nodes was
centrifuged on Ficoll-Isopaque to separate mononuclear
cells from erythrocytes. The resulting cell preparation
25   was washed extensively in PBS and incubated with
Dynabeads coated with anti-mouse IgG to remove B-
lymphocytes. The cells from this preparation were then
cultured in the presence of various concentrations of the
test antigen, as well as a non-related control antigen.
30   Cell proliferation was quantitated by measuring the
incorporation into the cells of radiolabelled thymidine
and or by the use of Promega Cell Titer 96 AQ kit. Again
efficacy of the T cell epitope analogues was
demonstrated.

35   Antibody responses to synthetic peptides
representing the immunodominant B-cell epitope H-(Asn-
Ala-Asn-Pro)$_3$-OH (SEQ ID NO: 23) of the circumsporozoite
protein were measured following intraperitoneal injection

- 14 -

of Balb/c mice. One hundred microgrammes of B-cell
epitope were administered in an equal volume of Freund's
complete adjuvant either alone or in a mixture (1:1) with
either noMalCST3 (SEQ ID NO: 5) or riMalCST3. As a
5 negative control, a further group of mice were immunised
with either noMalCST3 (SEQ ID NO: 5) or riMalCST3 in the
absence of the B-cell epitope. Three weeks after
priming, mice were boosted by the same route and with the
same dose of peptide in incomplete Freund's adjuvant. A
10 second booster injection was given two weeks after the
first with the same dose of antigen in incomplete
Freund's adjuvant. Blood samples were taken five days
later by retro-ocular bleeding and, after centrifugation,
the sera was immediately used in an enzyme-linked
15 immunosorbent assay (ELISA). Titres of antibodies
against the B-cell epitope were determined in microtitre
plates coated overnight at 4°C with 0.5 microgrammes of
synthetic peptide cross-linked to ovalbumin.

Low titre of antibodies were measured in mice
20 immunised with the B-cell epitope alone, however, much
higher titre of antibodies was observed in each case in
mice co-immunised with the same peptide and either form
of the T-cell epitope (Fig.2). All together, these
findings demonstrate the potential usefulness of
25 riMalCST3 and inMalCST3 as vaccine components; the
cellular immune response they elicit is responsive to the
normal antigen.

Antibody response to the same B-cell epitope was
also measured using five more T-cell epitopes selected
30 from the literature and synthesized in the following
forms:

<u>Malaria circumsporozoite protein:</u>

noMalCSA (Good *et al*, 1987):

H-Pro-Ser-Asp-Lys-His-Ile-Glu-Gln-Tyr-Leu-Lys-Lys-Ile-
35 Lys-Asn-Ser-Ile-Ser-NH$_2$ (SEQ ID NO: 3)

riMalCSA:

H-ser-ile-ser-asn-lys-ile-lys-lys-leu-tyr-gln-glu-ile-
his-lys-asp-ser-pro-NH$_2$

noMalCSB (Good *et al*, 1988):

H-His-Ile-Glu-Gln-Tyr-Leu-Lys-Lys-Ile-Lys-Asn-Ser-Ile-

Ser-NH$_2$ (SEQ ID NO: 4)

riMalCSB:

5   H-ser-ile-ser-asn-lys-ile-lys-lys-leu-tyr-gln-glu-ile-

his-NH$_2$

Diphtheria toxin:

noDipT (Bixler *et al*, 1989)

H-Gln-Val-Val-His-Asn-Ser-Tyr-Asn-Arg-Pro-Ala-Tyr-Ser-

10   Pro-Gly-NH$_2$ (SEQ ID NO:1)

riDipT:

H-Gly-pro-ser-tyr-ala-pro-arg-asn-tyr-ser-asn-his-val-

val-gln-NH$_2$

Pertussis toxin:

15   noPertT (Kim *et al*, 1990) (SEQ ID NO: 2):

H-His-Arg-Met-Gln-Glu-Ala-Val-Glu-Ala-Glu-Arg-Ala-Gly-

Arg-NH$_2$

riPertT:

H-arg-Gly-ala-arg-glu-ala-glu-val-ala-glu-gln-met-arg-

20   his-NH$_2$

Ovalbumin:

noOvalT (Sette *et al*, 1989) (SEQ ID NO: 7):

H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-

Glu-NH$_2$

25   riOvalT:

H-glu-asn-ile-glu-ala-his-ala-ala-his-val-ala-gln-ser-

ile-NH$_2$

The synthesis of the above peptides was performed
on Polyhipe Rink resin. The side chain protecting groups
30   used were: t-butyl for serine, threonine, aspartic acid,
glutamic acid and tyrosine; trityl for histidine,
glutamine and asparagine; t-butoxycarbonyl for lysine and
2,2,5,7,8-pentamethyl chroman-6-sulphonyl for arginine.
For diphtheria and pertussis peptides, cleavage and side-
35   chain deprotection were accomplished by reaction of the
peptidyl resins for 90 min at 0°C with 1M
trimethylsilylbromide-thioanisole in TFA containing 0.25M
1,2-ethanedithiol (5% v/v) and water (5% v/v) in TFA at

room temperature for 90 min.

In each case the mice developed very low titres against the B-cell epitope when immunised with the B-cell epitope alone, but produced much higher antibody titre when a mixture of the B-cell epitope and any of the T-cell epitopes in either no- or ri- form were used in the immunogen formulation (Fig. 3-7).

INDUSTRIAL APPLICATION

T cell epitope analogues in accordance with the invention have a range of potential applications in eliciting immunogenic responses in a host. These analogues can be used in the treatment and/or prophylaxis of diseases, and therapy of disease states. In particular, these analogues can be used in vaccines in animals, including humans for protection against pathogens and the like.

- 17 -

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10   F.V. Chisari (1986) T and B cell recognition of native and synthetic pre-S region determinants on HBsAg. In *Modern Approaches to Vaccines*. R. Chanock, R.A. Lerner and F Brown, eds. Cold Spring Harbor Laboratories, New York.

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30   insulin are differentially recognized by T but not by B cells in insulin immune guinea pigs. *J. Immunol.* <u>126</u>, 1095.

- 21 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i)   APPLICANT: Deakin Research Limited, N/A N/A
                                Comis, Alfio
                                Fischer, Peter
                                Tyler, Margaret I

    (ii)   TITLE OF INVENTION: EPITOPES

    (iii)   NUMBER OF SEQUENCES: 23

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              (F) ZIP: 2060

    (v)   COMPUTER READABLE FORM:
              (A) MEDIUM TYPE: Floppy disk
              (B) COMPUTER: IBM PC compatible
              (C) OPERATING SYSTEM: PC-DOS/MS-DOS
              (D) SOFTWARE: PatentIn Release #1.0,
                     Version #1.25

    (vi)   CURRENT APPLICATION DATA:
              (A) APPLICATION NUMBER: AU PM 4119
              (B) FILING DATE: 25-FEB-1994
              (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
              (A) NAME: Kurts, Ann D
              (B) REGISTRATION NUMBER: N/A
              (C) REFERENCE/DOCKET NUMBER: P21192

```
        (ix)    TELECOMMUNICATION INFORMATION:
                (A)  TELEPHONE: 61 2 957 5944
                (B)  TELEFAX: 61 2 957 6288
                (C)  TELEX: AA26547


5   (2)  INFORMATION FOR SEQ ID NO:1:


        (i)     SEQUENCE CHARACTERISTICS:
                (A)  LENGTH: 15 amino acids
                (B)  TYPE: amino acid
                (C)  STRANDEDNESS: single
10              (D)  TOPOLOGY: linear


        (ii)    MOLECULE TYPE: peptide


        (iii)   HYPOTHETICAL: NO


        (iv)    ANTI-SENSE: NO


        (v)     FRAGMENT TYPE: internal


15      (vi)    ORIGINAL SOURCE:
                (A)  ORGANISM: Corynebacterium diphtheriae


        (xi)    SEQUENCE DESCRIPTION: SEQ ID NO:1:


        Gln Val Val His Asn Ser Tyr Asn Arg Pro Ala Tyr Ser
        1               5                   10
20      Pro Gly
            15


    (2)  INFORMATION FOR SEQ ID NO:2:


        (i)     SEQUENCE CHARACTERISTICS:
                (A)  LENGTH: 14 amino acids
25              (B)  TYPE: amino acid
                (C)  STRANDEDNESS: single
                (D)  TOPOLOGY: linear
```

        (ii)   MOLECULE TYPE: peptide

        (iii)   HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

        (v)   FRAGMENT TYPE: internal

 5   (vi)   ORIGINAL SOURCE:
                (A) ORGANISM: Bordetella pertussis

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:2:

        His Arg Met Gln Glu Ala Val Glu Ala Glu Arg Ala Gly
        1   5   10
10   Arg

    (2) INFORMATION FOR SEQ ID NO:3:

        (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 18 amino acids
                (B) TYPE: amino acid
15   (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: peptide

        (iii)   HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

20   (v)   FRAGMENT TYPE: internal

        (vi)   ORIGINAL SOURCE:
                (A) ORGANISM: Plasmodium falciparum

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Ser Asp Lys His Ile Glu Gln Tyr Leu Lys Lys Ile
1   5   10
Lys Asn Ser Ile Ser
            15

(2) INFORMATION FOR SEQ ID NO:4:

    (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 14 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii)   MOLECULE TYPE: peptide

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (v)   FRAGMENT TYPE: internal

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:4:

His Ile Glu Gln Tyr Leu Lys Lys Ile Lys Asn Ser Ile
1   5   10
Ser

(2) INFORMATION FOR SEQ ID NO:5:

    (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: peptide

        (iii)   HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

        (v)   FRAGMENT TYPE: internal

5   (vi)   ORIGINAL SOURCE:
                  (A) ORGANISM: Plasmodium falciparum

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:5:

        Gly Asp Ile Glu Lys Lys Ile Ala Lys Met Glu Lys Ala
        1   5   10
10   Ser Ser Val Phe Asn Val Val Asn Ser
              15   20

    (2) INFORMATION FOR SEQ ID NO:6:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 16 amino acids
15   (B) TYPE: amino acid
                  (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: peptide

        (iii)   HYPOTHETICAL: NO

20   (iv)   ANTI-SENSE: NO

        (v)   FRAGMENT TYPE: internal

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:6:

Cys Ser Ala Leu Leu Ser Ser Asp Ile Thr Ala Ser Val
1   5   10
Asn Cys Ala
        15

(2) INFORMATION FOR SEQ ID NO:7:

    (i)   SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 14 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

    (ii)   MOLECULE TYPE: peptide

   (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (v)   FRAGMENT TYPE: internal

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ile Ser Gln Ala Val His Ala Ala His Ala Glu Ile Asn
1   5   10
Glu

(2) INFORMATION FOR SEQ ID NO:8:

    (i)   SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 14 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

(ii)   MOLECULE TYPE: peptide

(iii)   HYPOTHETICAL: NO

(iv)   ANTI-SENSE: NO

(v)   FRAGMENT TYPE: internal

5   (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:8:

Tyr Thr Tyr Thr Val His Ala Ala His Ala Tyr Thr Tyr
1   5   10
Thr

(2) INFORMATION FOR SEQ ID NO:9:

10   (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 amino acids
                (B) TYPE: amino acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

15   (ii)   MOLECULE TYPE: peptide

        (iii)   HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

        (v)   FRAGMENT TYPE: internal

        (vi)   ORIGINAL SOURCE:
20   (A) ORGANISM: Measles virus

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Ile Leu Glu Ser Arg Gly Ile Lys Ala Arg Ile Thr
1   5   10
His Val Asp Thr Glu Ser Tyr
        15   20

(2) INFORMATION FOR SEQ ID NO:10:

    (i)   SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 15 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

    (ii)   MOLECULE TYPE: peptide

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (v)   FRAGMENT TYPE: internal

    (vi)   ORIGINAL SOURCE:
             (A) ORGANISM: Measles virus

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Ser Glu Ile Lys Gly Val Ile Val His Arg Leu Glu
1   5   10
Gly Val
        15

(2) INFORMATION FOR SEQ ID NO:11:

    (i)   SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 16 amino acids
             (B) TYPE: amino acid

          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii)   MOLECULE TYPE: peptide

    (iii)   HYPOTHETICAL: NO

5   (iv)   ANTI-SENSE: NO

    (v)   FRAGMENT TYPE: internal

    (vi)   ORIGINAL SOURCE:
          (A) ORGANISM: Respiratory syncytial virus

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:11:

10   Cys Glu Tyr Asn Val Phe His Asn Lys Thr Phe Glu Leu
      1   5   10
    Pro Arg Ala
         15

   (2) INFORMATION FOR SEQ ID NO:12:

15   (i)   SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 11 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

20   (ii)   MOLECULE TYPE: peptide

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (v)   FRAGMENT TYPE: internal

(vi)   ORIGINAL SOURCE:
         (A) ORGANISM: Influenza virus

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ser Ser Phe Glu Arg Phe Glu Ile Phe Pro Lys
1   5   10

(2) INFORMATION FOR SEQ ID NO:13:

     (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 11 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

     (ii)   MOLECULE TYPE: peptide

     (iii)   HYPOTHETICAL: NO

     (iv)   ANTI-SENSE: NO

     (v)   FRAGMENT TYPE: internal

     (vi)   ORIGINAL SOURCE:
              (A) ORGANISM: Influenza virus

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Val Thr Ala Ala Cys Ser His Glu Gly Lys
1   5   10

(2) INFORMATION FOR SEQ ID NO:14:

     (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 12 amino acids
              (B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii)   MOLECULE TYPE: peptide

(iii)   HYPOTHETICAL: NO

5   (iv)   ANTI-SENSE: NO

(v)   FRAGMENT TYPE: internal

(vi)   ORIGINAL SOURCE:

(A) ORGANISM: Influenza virus

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:14:

10 Cys Pro Lys Tyr Val Arg Ser Ala Lys Leu Arg Met

    1   5   10

(2) INFORMATION FOR SEQ ID NO:15:

(i)   SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

15   (B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii)   MOLECULE TYPE: peptide

(iii)   HYPOTHETICAL: NO

20 (iv)   ANTI-SENSE: NO

(v)   FRAGMENT TYPE: internal

(vi)   ORIGINAL SOURCE:

(A) ORGANISM: pig

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:15:

Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr
1   5   10

(2) INFORMATION FOR SEQ ID NO:16:

5   (i)   SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 12 amino acids
                  (B) TYPE: amino acid
                  (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear

10   (ii)   MOLECULE TYPE: peptide

         (iii)   HYPOTHETICAL: NO

         (iv)   ANTI-SENSE: NO

         (v)   FRAGMENT TYPE: internal

         (vi)   ORIGINAL SOURCE:
15   (A) ORGANISM: pig

         (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1   5   10

(2) INFORMATION FOR SEQ ID NO:17:

20   (i)   SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 13 amino acids
                  (B) TYPE: amino acid
                  (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear

25   (ii)   MOLECULE TYPE: peptide

(iii)   HYPOTHETICAL: NO

(iv)   ANTI-SENSE: NO

(v)   FRAGMENT TYPE: internal

(vi)   ORIGINAL SOURCE:
5   (A) ORGANISM: Hepatitis B virus

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Gln Trp Asn Ser Thr Thr Phe His Gln Thr Leu Gln
1   5   10

(2) INFORMATION FOR SEQ ID NO:18:

10   (i)   SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 15 amino acids
                  (B) TYPE: amino acid
                  (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear

15   (ii)   MOLECULE TYPE: peptide

(iii)   HYPOTHETICAL: NO

(iv)   ANTI-SENSE: NO

(v)   FRAGMENT TYPE: internal

(vi)   ORIGINAL SOURCE:
20   (A) ORGANISM: Hepatitis B virus

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Leu Asn Phe Leu Gly Gly Thr Thr Val Cys Leu Gly
1   5   10
Gln Asn
        15

(2) INFORMATION FOR SEQ ID NO:19:

    (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 15 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii)   MOLECULE TYPE: peptide

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (v)   FRAGMENT TYPE: internal

    (vi)   ORIGINAL SOURCE:
            (A) ORGANISM: Hepatitis B virus

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Val Leu Leu Asp Tyr Gln Gly Met Leu Pro Val Cys
1   5   10
Pro Leu
        15

(2) INFORMATION FOR SEQ ID NO:20:

    (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 15 amino acids
            (B) TYPE: amino acid

                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

          (ii)   MOLECULE TYPE: peptide

          (iii)   HYPOTHETICAL: NO

5   (iv)   ANTI-SENSE: NO

          (v)   FRAGMENT TYPE: internal

          (vi)   ORIGINAL SOURCE:
                    (A) ORGANISM: Hepatitis B virus

          (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:20:

10   Thr Lys Pro Ser Asp Gly Asn Cys Thr Cys Ile Pro Ile
       1   5   10
       Pro Ser
             15

     (2) INFORMATION FOR SEQ ID NO:21:

15   (i)   SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 20 amino acids
                    (B) TYPE: amino acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

20   (ii)   MOLECULE TYPE: peptide

          (iii)   HYPOTHETICAL: NO

          (iv)   ANTI-SENSE: NO

          (v)   FRAGMENT TYPE: internal

(vi)   ORIGINAL SOURCE:
         (A) ORGANISM: Foot and mouth disease virus

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:21:

Val Pro Asn Leu Arg Gly Asp Leu Gln Val Leu Ala Gln
1   5   10
Lys Val Ala Arg Thr Leu Pro
        15   20

(2) INFORMATION FOR SEQ ID NO:22:

     (i)   SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 13 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

     (ii)   MOLECULE TYPE: peptide

     (iii)   HYPOTHETICAL: NO

     (iv)   ANTI-SENSE: NO

     (v)   FRAGMENT TYPE: internal

     (vi)   ORIGINAL SOURCE:
             (A) ORGANISM: Rabies virus

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:22:

Asp Glu Gly Cys Thr Asn Leu Ser Gly Phe Ser Tyr Met
1   5   10

(2) INFORMATION FOR SEQ ID NO:23:

        (i)   SEQUENCE CHARACTERISTICS:
                 (A) LENGTH: 4 amino acids
                 (B) TYPE: amino acid
5   (C) STRANDEDNESS: single
                 (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: peptide

        (iii)   HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

10   (v)   FRAGMENT TYPE: internal

        (vi)   ORIGINAL SOURCE:
                 (A) ORGANISM: Plasmodium falciparum

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:23:

        Asn Ala Asn Pro
15   1

CLAIMS

1.   A synthetic peptide T cell epitope analogue
of a native T cell epitope which analogue is partially or
completely inverso modified with respect to the native T
cell epitope.
2.   A synthetic peptide T cell epitope analogue
of a native T cell epitope which analogue is partially or
completely retro-inverso modified with respect to the
native T cell epitope.
3.   A synthetic peptide T cell epitope analogue
acoording to claim 1 or claim 2 wherein the native T cell
epitope is selected from the group consisting of:
H-Gln-Val-Val-His-Asn-Ser-Tyr-Asn-Arg-Pro-Ala-Tyr-Ser-
Pro-Gly-OH, from diphtheria toxin (SEQ ID NO: 1);
H-His-Arg-Met-Gln-Glu-Ala-Val-Glu-Ala-Glu-Arg-Ala-Gly-
Arg-OH, from pertussis toxin (SEQ ID NO: 2);
H-Pro-Ser-Asp-Lys-His-Ile-Glu-Gln-Tyr-Leu-Lys-Lys-Ile-
Lys-Asn-Ser-Ile-Ser-OH, from malaria CSA protein (SEQ ID
NO: 3);
H-His-Ile-Glu-Gln-Tyr-Leu-Lys-Lys-Ile-Lys-Asn-Ser-Ile-
Ser-OH, from malaria CSB protein (SEQ ID NO: 4);
H-Gly-Asp-Ile-Glu-Lys-Lys-Ile-Ala-Lys-Met-Glu-Lys-Ala-
Ser-Ser-Val-Phe-Asn-Val-Val-Asn-Ser-OH, from malaria CST3
protein (SEQ ID NO: 5);
H-Cys-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-
Asn-Cys-Ala-OH, from hen egg lysozyme (SEQ ID NO: 6);
H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-
Glu-OH (SEQ ID nO: 7) and
H-Tyr-Thr-Tyr-Thr-Val-His-Ala-Ala-His-Ala-Tyr-Thr-Tyr-
Thr-OH (SEQ ID NO: 8), from ovalbumin;
MVF:258-277 H-Gly-Ile-Leu-Glu-Ser-Arg-Gly-Ile-Lys-Ala-
            Arg-Ile-Thr-His-Val-Asp-Thr-Glu-Ser-Tyr-OH
            (SEQ ID NO: 9)
and
MVF:288-302 H-Leu-Ser-Glu-Ile-Lys-Gly-Val-Ile-Val-His-
            Arg-Leu-Glu-Gly-Val-OH (SEQ ID NO: 10), from
            measles virus F and H glycoproteins;

RS1A:45-60   H-Cys-Glu-Tyr-Asn-Val-Phe-His-Asn-Lys-Thr-
             Phe-Glu-Leu-Pro-Arg-Ala-OH (SEQ ID NO: 11),
             from respiratory syncytial virus 1A protein;

Influenza hamagglutinin A/PR/8/34 Mt.S.: residues 109-119
(SEQ ID NO: 12), 130-140 (SEQ ID NO: 13), and 302-313
(SEQ ID NO: 14);

residues (A)4-14 (SEQ ID NO: 15) and (B)5-16 (SEQ ID NO:
16) from pork insulin;

Hepatitis B virus pre S residues 120-132 (SEQ ID NO: 17);

Hepatitis B virus major surface antigen: residues 38-52
(SEQ ID NO: 18), 95-109 (SEQ ID NO: 19), and 140-154 (SEQ
ID NO: 20);

Foot and mouth virus VP1: residues 141-160 (SEQ ID NO:
21); and

Rabies virus-spike glycoprotein precursor: residues 32-44
(SEQ ID NO: 22).

        4.   A T cell epitope analogue according to claim
2 or claim 3 wherein the amino acid residues flanking the
retro-inverted sequence are substituted by
side-chain-analogous α-substituted
geminal-diaminomethanes and malonates.

        5.   A vaccine comprising a T cell epitope
analogue according to any one of claims 1 to 4 together
with a B cell epitope and a pharmaceutically acceptable
carrier, diluent, excipient and/or adjuvant.

        6.   A vaccine according to claim 5 wherein the T
cell epitope analogue is conjugated to the B cell
epitope.

        7.   A vaccine according to claim 5 which is a
cocktail of T cell epitope analogues and B cell epitopes
tailored to the condition against which vaccination is
required.

        8.   A vaccine according to claim 5 wherein the B
cell epitope is a peptide or polypeptide of any length
whose amino acid sequences stem from:

polypeptides of a pathogen including poliomyelitis,
hepatitis B, foot and mouth disease of livestock,
tetanus, pertussis, HIV, cholera, malaria, influenza,

rabies or diphtheria causing agents;

a toxin including robustoxin, heat labile toxin of

pathogenic *Escherichia coli* strains and Shiga toxin from

Shigella dysenteriae;

Amyloid ß protein;

human chorionic gonadotropin;

or gonadotropin releasing hormone.

9.   A vaccine according to claim 5 wherein the B cell

epitope is a retro, retro-inverso or inverso antigen

analogue.

10. A method of vaccinating a host in need of such

treatment which method comprises administering an

effective amount of a vaccine according to claim 5 to the

host.

11.   A method of preparing a T cell epitope

analogue according to claim 1 or 2, the method comprising

synthesising a partially or completely inverso or retro-

inverso analogue of the native T cell epitope.

12. Antibodies produced by immunisation of a host

with a vaccine according to claim 5.

13.   A method of preparing a vaccine according to

claim 5 which method comprises: conjugating a T cell

epitope analogue according to claim 1 or claim 2 to a B

cell epitope, or admixing a T cell epitope analogue

according to claim 1 or claim 2 with a B cell epitope;

and admixing an effective amount of the resulting mixture

or conjugate with a pharmaceutically or veterinarally

acceptable carrier, diluent, excipient and/or adjuvant.

FIG. 1

1/TITRE	CONTROL ■	NORMAL □	ANALOGUE ◆
2	0.84	1.425	1.32
4	0.555	1.89	1.585
8	0.465	1.825	1.775
16	0.24	0.84	1.16
32	0.265	0.9	0.935
64	0.08	0.74	0.815
128	0.09	0.625	0.555
256	0.06	0.305	0.335
512	0.02	0.23	0.14
1024	0	0.095	0.07
2048	0.005	0.095	0.06

FIG.2

FIG.3

1/TITRE	CONTROL ■	NORMAL □	ANALOGUE ♦
5	0.733	1.343	2.667
10	0.651	0.98	2.317
20	0.481	0.726	1.266
40	0.266	0.551	1.231
80	0.232	0.42	0.712
160	0.126	0.277	0.612
320	0.123	0.194	0.561
640	0.071	0.11	0.456
1280	0.061	0.104	0.317
2560	0.026	0.102	0.231
5120	0.023	0.101	0.126

FIG.4

1/TITRE	CONTROL ■	NORMAL □	ANALOGUE ◆
5	0.806	1.567	2.96
10	0.2	1.252	2.7
20	0.175	0.692	1.534
40	0.06	0.682	0.951
80	0.056	0.495	0.854
160	0.041	0.697	0.629
320	0.035	0.239	0.54
640	0.017	0.095	0.44
1280	0.006	0.072	0.35
2560	0.005	0.061	0.29
5120	0.001	0.027	0.164

FIG.5

1/TITRE	CONTROL ■	NORMAL □	ANALOGUE ◆
5	0.967	2.63	2.887
10	0.821	1.76	2.438
20	0.66	1.65	2.335
40	0.416	0.832	1.86
80	0.411	0.775	1.364
160	0.284	0.727	0.934
320	0.242	0.619	0.795
640	0.193	0.434	0.652
1280	0.109	0.2	0.53
2560	0.098	0.074	0.38
5120	0.097	0.069	0.177

FIG.6

1/TITRE	CONTROL ■	NORMAL □	ANALOGUE ◆
5	0.868	2.823	1.354
10	0.85	1.395	1.363
20	0.837	1.351	1.284
40	0.65	1.425	0.844
80	0.38	0.775	0.615
160	0.271	0.643	0.554
320	0.266	0.603	0.42
640	0.163	0.378	0.196
1280	0.084	0.271	0.253
2560	0.076	0.211	0.157
5120	0.073	0.192	0.096

FIG.7

1/TITRE	CONTROL ■	NORMAL □	ANALOGUE ◆
5	0.991	2.375	2.646
10	0.51	1.473	1.373
20	0.351	1.217	0.832
40	0.11	0.663	0.645
80	0.092	0.595	0.644
160	0.054	0.186	0.412
320	0.026	0.158	0.214
640	0.023	0.149	0.191
1280	0.006	0.087	0.133
2560	0.005	0.072	0.087
5120	0.003	0.071	0.074

A.	CLASSIFICATION OF SUBJECT MATTER

Int. Cl.^{6}
C07K14/705,14/34,14/235,14/445,14/165,14/77,14/12,14/135,14/11,14/62,14/02,14/09,14/145,16/28,16/08,16/10,
16/12,16/18,16/20,16/26; A61K 39/00,39/015,39/05,39/10,39/145,39/135,39/165,39/205,39/29,39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: as above using Derwent (WPAT) and keywords.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU:IPC: as above.

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
Derwent (WPAT): Retro or Inverso or Retro( ) Inverso and T-cell
Chemical Abstracts: Retro or Inverso or Retro ( ) Inverso and not Retrovirus or Retroviral or Retrobulbar and T-cell

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	AU 49346/93 A (DEAKIN RESEARCH LIMITED) 29 March 1994 whole document, especially Examples 6, 7, 14-17, Claims 7-9,12	1-4,12
A	ANGEWANDTE CHEMIE Vol. 31, No. 6 (1992) DUERR, Hansjoerg, et al. 'Retro-inverso amide bonding between trifunctional amino acids' whole document	1-4

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

*	Special categories of cited documents :	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 May 1995	5 JUNE 1995 (05.06.95)

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(54) Title: ANTIMICROBIAL OIL-IN-WATER EMULSIONS

(57) Abstract

An antimicrobial oil-in-water emulsion comprising an agent selected from the group consisting of glycerol monooleate, glycerol trioleate, glycerol monolaurate, and glycerol dilaurate as the primary emulsifier and a cationic halogen-containing compound having a C_{12}-C_{16} chain as a positive charge producing agent is disclosed. The emulsion can optionally further include a sterol, preferably phytosterol. Preferred cationic halogen-containing compounds having a C_{12}-C_{16} chain for use in the invention include cetylpyridinium chloride, cetylpyridinium bromide, cetyltrimethylammonium bromide, cetyldimethyethylammonium bromide, and benzalkonium chloride. The antimicrobial emulsion can be used in the form of a pharmaceutical preparation to inhibit the growth of a wide variety of infectious pathogens, including bacteria, fungi, and viruses.

Antimicrobial Oil-In-Water Emulsions

Background of the Invention

5   The present invention relates to an antimicrobial lipid-containing oil-in-water emulsion which inactivates infectious pathogens upon contact.

It is known that if a water-immiscible liquid phase is mixed into an aqueous phase by mechanical agitation, for example, by means of an ultra-disperser, the stability of the
10   resulting oil-in-water dispersion most frequently requires the addition of an emulsifying agent, the molecules of which are adsorbed onto the surface of the oil droplets to form a kind of continuous membrane which prevents direct contact between two adjacent droplets. The drops of oil can further contain substances soluble in an organic medium, such as a sterol.

15   In addition to discrete oil droplets dispersed in an aqueous phase, oil-in-water emulsions can also contain other lipid structures, such as small lipid vesicles (i.e., lipid spheres which often consist of several substantially concentric lipid bilayers separated from each other by layers of aqueous phase), micelles (e.g., amphiphile molecules in small clusters of 50-200 molecules arranged so that the polar head groups face outward toward the aqueous
20   phase and the apolar tails are sequestered inward away from the aqueous phase), or lamellar phases (lipid dispersions in which each particle consists of parallel glycerol ester bilayers separated by thin films of water). These lipid structures are formed as a result of hydrophobic forces which drive apolar residues (i.e., long hydrocarbon chains) away from water.

25   The portals of entry of pathogenic bacteria, viruses or fungi are predominantly the skin and mucus membranes, and upper and lower respiratory tracts. The first step in any infection is attachment or colonization on skin or mucus membranes with subsequent invasion and dissemination of the infectious pathogen. Accordingly, an object of the present invention is to provide an antimicrobial emulsion which inactivates infectious pathogens on
30   contact by disrupting their membrane structures.

Summary of the Invention

The present invention provides a stable antimicrobial oil-in-water emulsion for inactivating infectious pathogens upon contact. The emulsion comprises positively charged
35   droplets of a lipid-containing oily "discontinuous phase" dispersed in an aqueous "continuous phase". It is believed that the oily discontinuous phase inactivates infectious pathogens by disrupting their membrane structure. The emulsion has microbicidal activity against a broad spectrum of bacteria, viruses and yeasts.

The antimicrobial emulsion of the present invention consists primarily of positively charged droplets of an oily discontinuous phase dispersed in an aqueous continuous phase, such as water. The discontinuous phase contains a glycerol ester selected from the group consisting of glycerol monooleate (GMO), glycerol trioleate (GTO), glycerol monolaurate GMO), and glycerol dilaurate (GDL) and a cationic halogen-containing compound, preferably one having a C_{12}-C_{16} chain as a positive charge producing agent. The droplets can further contain a sterol, such as cholesterol or phytosterol. The droplets appear to bind to negatively charged proteins contained in bacterial, viral, or fungal membranes, thereby disrupting the membrane structure and irradiating the pathogen.

Antimicrobial emulsions of the present invention are non-toxic and safe, for example, when swallowed, inhaled, or applied to the skin. This result is unexpected since many cationic halogen-containing compounds having a C_{12}-C_{16} chain are extremely toxic if administered alone. For example, cetylpyridinium chloride (CPC), the preferred cationic halogen-containing compound of the invention, causes severe irritation and damage to tissues of the upper respiratory tract, mucous membranes and skin. However, when administered in the form of an emulsion of the invention, no such adverse effects occur. Furthermore, the emulsions of the invention are stable when heated or exposed to significant levels of acid and base.

The positive charge of the oily discontinuous phase is provided by a cationic halogen-containing compound having a C_{12}-C_{16} chain. In a preferred embodiment, the cationic halogen-containing compound having a C_{12}-C_{16} chain is selected from the group consisting of cetylpyridinium chloride (CPC), cetylpyridinium bromide (CPB), cetyltrimethylammonium bromide (CTAB), and cetyldimethyethylammonium bromide (CDEAB). Other cationic halogen-containing compounds having a C_{12}-C_{16} chain which can be used include, for example, cetyltrimethylammonium chloride, cetylbenzyldimethylammonium chloride, cetyltributylphosphonium bromide, dodecyltrimethylammonium bromide, and tetradecyltrimethylammonium bromide.

The oily discontinuous phase can further contain at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof. The term "cholesterol derivatives," as used herein, includes but is not limited to sulfate and phosphate derivatives of cholesterol. Preferred sterols include phytosterols, such as soya sterol.

Oils useful in forming antimicrobial oil-in-water emulsions of the present invention include a broad spectrum of water-immiscible materials, such as soybean oil, avocado oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower

oil, fish oils, flavor oils, water insoluble vitamins, and mixtures thereof. In a preferred
embodiment of the invention, the oil is a scented or flavored oil such as peppermint oil.

In another embodiment of the invention, at least a portion of the emulsion may be in
the form of lipid structures including, but not limited to, unilamellar, multilamellar, and
paucilamellar lipid vesicles, micelles, and lamellar phases.

The antimicrobial emulsions of the present invention can be used, for example, in
pharmaceutical preparations (e.g., creams, solutions and suspensions) to inhibit the growth of
a wide variety of infectious pathogens, including bacteria, viruses, and fungi. Accordingly,
the present invention also provides an antimicrobial preparation suitable for pharmaceutical
administration made up of an antimicrobial emulsion of the invention and a pharmaceutically
acceptable carrier. The preparation can be applied topically to skin surface areas, mucus
membranes, or oral surfaces, for example, as a cream, gel, spray, mouthwash, or deodorant to
treat or prevent bacterial infections such as *Propionibacterium acnes, Neisseria gonorrhea,
Streptococcus, and Staphylococcus epidermidis*. Alternatively, the preparation can be
administered internally by, for example, oral or intravenous administration. Such systemic
administration of the preparation can be used, for example, to inactivate pathogenic
microorganisms, e.g., bacteria such as *Helicobacter pylori*, and viruses, particularly envelope
viruses. In a preferred embodiment of the invention, the preparation is administered orally to
an individual to treat infection by HIV.

Accordingly, the present invention further provides a method for inhibiting the
growth of an infectious pathogen by topical or systemic administration of the antimicrobial
emulsion of the invention.

Detailed Description of the Invention

The present invention relates to a stable antimicrobial oil-in-water emulsion made of
positively charged droplets of an oily discontinuous phase dispersed in an aqueous
continuous phase.

The term "antimicrobial," as used herein, means having the ability to inactivate
infectious pathogens. The term "inactivate", as used herein, includes but is not limited to,
killing or inhibiting growth of the organism. The term "infectious pathogen", as used herein,
includes, but is not limited to, fungi, viruses, bacteria, and parasites. The antimicrobial
emulsion of the present invention inactivates a wide variety of infectious pathogens. It
appears that inactivation is achieved by disruption of the membrane structure of the pathogen
by the components of the oily discontinuous phase.

The term "emulsion," as used herein, includes both classic oil-in-water dispersions or droplets, as well as other lipid structures which can form as a result of hydrophobic forces which drive apolar residues (i.e., long hydrocarbon chains) away from water and drive polar

5   head groups toward water, when a water immiscible oily phase is mixed with an aqueous phase. These other lipid structures include, but are not limited to, unilamellar, paucilamellar, and multilamellar lipid vesicles, micelles, and lamellar or hexagonal phases. These other lipid structures also contain a glycerol ester selected from the group consisting of glycerol monooleate (GMO), glycerol trioleate (GTO), glycerol monolaurate (GML), and glycerol

10 dilaurate (GDL) as the primary emulsifier, and a dodacyl to hexadecyl cationic halogen-containing compound as a positive charge producing agent. These other lipid structures can also further include at least one sterol, preferably a phytosterol. In a preferred embodiment of the invention, GMO is used as the primary emulsifier. The term "primary emulsifier", as used herein, refers to the emulsifier which constitutes the greatest proportion by weight of

15 any single emulsifier contained in the oily discontinuous phase.

Antimicrobial oil-in-water emulsions of the present invention can be formed using classic emulsion forming techniques which are well known in the art. In brief, the lipid-oil phase is mixed with the aqueous phase under relatively high shear forces to obtain an oil-in-

20 water emulsion containing oil droplets which are approximately 1 micron in diameter. More particularly, a positively charged lipid-containing oily discontinuous phase is formed by blending (a) an oil carrier; (b) a glycerol ester selected from the group consisting of GMO, GTO, GML, or GDL; and (c) a cationic halogen-containing compound having a C_{12}-C_{16} chain, along with any other compatible glycerol esters or emulsifiers, such as Polysorbate 60,

25 and any sterols or other lipophilic materials to be incorporated into the lipid-oil phase.

Once the lipid-oil phase is formed, it is heated and blended with an aqueous phase (e.g., water, saline, or any other aqueous solution which can hydrate the lipids) on a volume to volume basis ranging from about 1:4 to 1:2, preferably about 1:3 lipid-oil phase to aqueous

30 phase. The lipid-oil and aqueous phases can be blended using any apparatus capable of producing the high shear mixing forces, including, for example, a french press, a Novamix™ lipid vesicle maker (IGI Inc., Buena N.J.), a syringe mixer, or, alternatively by hand using two syringes as described in U.S. Patents Nos. 4,895,452 and 4,911,928, the teachings of which are incorporated by reference herein.

35

Antimicrobial oil-in-water emulsions of the present invention provide the advantage of being stable in the presence of heat, acid, or base. For example, as shown below in Example 7, emulsions of the invention are not significantly altered or broken down when

boiled or exposed to 1N nitric acid or 1N sodium hydroxide. This stability makes the emulsions suitable for pharmaceutical administration, even internal administration.

Antimicrobial oil-in-water emulsions of the present invention can be used to inactivate a variety of infectious pathogens upon contact. As described in the examples below, microbes which are inactivated by the present invention include a wide variety of bacteria, fungi and viruses. More specifically, for inactivation of bacteria which colonize in oral or oropharyngeal areas, such as *Streptococcus pneumoniae*, Group A beta-hemolytic *Streptococcus, Haemophilus influenzae*, and *Neisseria meningitidis*, the presently disclosed emulsions can be applied topically, as a spray or mouthwash. For bacteria which colonize in gastrointestinal areas, such as *Helicobacter pylori*, the emulsions can be administered orally, for example, as a pill or a liquid. For bacteria and fungi which colonize in the vagina, such as *Neisseria gonorrhoeae, Gardnerella vaginalis*, Group B *Streptococcus*, and *Candida albicans*, the emulsions can be applied topically as a cream, gel, or suppository. The presently disclosed emulsions can also be used for dermatological application as a cream or gel to inactivate or prevent infection secondary to *Propionibacterium acnes, Staphylococcus aureus, Staphylococcus epidermidis*, and Group B *Streptococcus*. In a preferred embodiment of the invention, antimicrobial emulsions of the present invention are used to prevent infection by gram positive bacteria.

Antimicrobial oil-in-water emulsions of the present invention can also be used to inactivate a variety of viruses, particularly enveloped viruses upon contact. For example, the presently disclosed emulsions can be used for oropharyngeal, oral, veneral, dermatological, or internal application to inactivate or prevent infection secondary to viruses including *Herpesviridae* (e.g., herpes simplex types 1 and 2, Epstein-Barr virus, cytomegalovirus, varicella virus, and human herpes virus type 6), *Togaviridae* (e.g., Rubella virus), *Flaviviridae*, (e.g., Yellow Fever virus), *Coronaviridae (e.g.,* Corona viruses*), Rhabdoviridae (e.g.,* Rabies virus*), Filoviridae (e.g.,* Marburg virus*), Paramyxoviridae (e.g.,* Measles virus*), Orthomyxoviridae (*e.g., Influenza viruses*), Bunyaviridae (e.g.,* California encephalitis virus*), Arenaviridae (e.g.,* Lymphocytic choriomeningitis virus*), Retroviridae (e.g., HIV-1)*, and *Hepadnavirida (*e.g., Hepatitis B*).* The emulsions of the invention can also be used to inactivate viruses in vitro, for example, to disinfect contaminated blood products. In a preferred embodiment of the invention, the oil-in-water emulsions are used to inactivate HIV.

The present invention also provides an antimicrobial preparation suitable for pharmaceutical administration consisting of the antimicrobial emulsion of the present invention and a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier," as used herein, refers to any physiologically compatible carrier for use in pharmaceutical administration. Use of such media and agents for pharmaceutically active

6

substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the emulsions of the present invention, use thereof in a pharmaceutical preparation is contemplated.

5   The present invention further provides methods for inhibiting the growth of an infectious pathogen by topical, or systemic administration of the antimicrobial emulsion of the present invention, preferably in the form of a pharmaceutical preparation. The term "topical," as used herein, includes application to mucous membranes, oral surfaces, skin, inner ear surfaces, or the surfaces of any bodily orifice, such as the vagina or rectum. The
10   term "systemic", as used herein, includes any form of internal administration, including but not limited to, oral and intravenous administration.

       The following examples will illustrate the efficacy of the invention.

15

EXAMPLES

Example 1 - Preparation of GMO and GMS Oil-in-Water Emulsions
       In this Example, a series of positively and negatively charged lipid-containing oil-in-
20   water emulsions having either GMO or GMS as the primary emulsifier were formed. Selected emulsions were also characterized with regard to purity, pH and size of oil droplets.

       Table 1 shows the amount of each chemical component used to form the lipid-oil phase of several charged GMO and GMS oil-in-water emulsions having glycerol monooleate
25   (GMO) or glycerol monostearate (GMS) as the primary emulsifiers, and cetylpyridinium chloride (CPC), cetylpyridinium bromide (CPB), cetyltrimethylammonium bromide (CTAB), or dimethyldioctadecylammonium bromide (DDDAB) as positive charge producing agents, or oleic acid as a negative charge producing agent.

TABLE 1

	GMO/ CPC	GMO/ CPB	GMO/ CTAB	GMS/ DDDAB	GMO/ OLEIC	GMS/ CPC
GMO	3.43 g	3.43 g	3.43 g	---------	3.43 g	--------
GMS	------	-----	-----	3.43 g	-----	3.43 g
Soya Sterol or cholesterol	0.96 g	0.96 g	0.96 g	0.96 g	0.96 g	0.96 g
Tween 60	0.84 g	0.84 g	0.84 g	0.84 g	0.84 g	0.84 g
Soybean Oil	11 g	11 g	11 g	11 g	11 g	11 g
CPC	130 mg	------	-----	------	------	130 mg
CPB	-----	150 mg	-----	-----	------	-----
CTAB	-----	-----	140 mg	-----	-----	-----
DDDAB	-----	-----	-----	240 mg	-----	-----
Oleic Acid	-----	-----	-----	-----	108 mg	-----

To prepare the charged emulsions shown above in Table 1, a lipid-oil phase containing approximately 12 to 21% by weight GMO or GMS, 0.8 to 0.9% by weight
5   cationic halogen-containing compound (or 0.7% by weight oleic acid), 67 to 80% by weight carrier oil such as soybean oil, 3 to 5% by weight Tween 60 (Polyoxyethylene 20 sorbitan monostearate), and 3 to 6% by weight soya sterol was heated for approximately one hour at 86°C. The lipid-oil phase was then blended with an aqueous phase containing water at 65°C using a 5 ml syringe machine as described in US Patent No. 4,895,452 on a volume to
10   volume basis of 13 parts lipid-oil to 37 parts water.

Table 2 shows the pH of the emulsions shown in Table 1. Also shown is the size of the lipid-oil droplets of the emulsions measured on a Coulter LS 130 Laser sizing instrument equipped with a circulating waterbath.
15

TABLE 2

Chemical Components of Emulsion	Charge	pH	Mean Coulter Size in Microns	Mean Coulter Range in Microns
GMO/CPC	Positive	3.72	1.049	0.720-1.401
GMO/CPB	Positive	4.31	0.891	0.680-1.124
GMO/CTAB	Positive	4.82	1.143	0.647-1.358
GMS/DDDAB	Positive	5.86	1.080	0.694-1.532
GMO/Oleic acid	Negative	4.45	1.078	0.738-1.448
GMS/CPC	Positive	3.72	1.047	0.677-1.497

Example 2 - Preparation of 10N GMO Oil-in-Water Emulsions

In this Example, a series of positively charged oil-in-water emulsions were prepared having GMO as the primary emulsifier and a 10 fold higher percentage (10N) of cationic
5   halogen-containing compound (cetylpyridinium chloride (CPC), cetylpyridinium bromide (CPB), cetyltrimethylammonium bromide (CTAB), cetyldimethyethylammonium bromide (CDEAB), and benzalkonium chloride (BAC)), as compared with Example 1, as a positive charge producing agent.

10   Table 3 shows the amount of each chemical component used to form the lipid-oil phase of the positively charged 10N GMO emulsions.

TABLE 3   Positively Charged 10N Emulsions

Chemical Components of Emulsion	GMO/ CPC	GMO/ CPB	GMO/ CTAB	GMO/ CDEAB	GMO/ BAC
GMO	3.43 g	3.43 g	3.43 g	3.43g	3.43 g
Soya Sterol or cholesterol	0.96 g	0.96 g	0.96 g	0.96 g	0.96 g
Tween 60	0.84 g	0.84 g	0.84 g	0.84 g	0.84 g
Soybean Oil	11 g	11 g	11 g	11 g	11 g
CPC	1.3 g	------	-----	------	------
CPB	-----	1.5 g	-----	-----	------
CTAB	-----	-----	1.4 g	-----	-----
CDEAB	-----	-----	-----	1.3 g	-----
BAC	-----	-----	-----	-----	1.3 g

15   To prepare the 10N emulsions shown above in Table 3, a lipid-oil phase containing approximately 19% by weight GMO, 62% by weight carrier oil such as soybean oil, 4.7% by weight Tween 60 (Polyoxyethylene 20 sorbitan monostearate), 5.4% by weight soya sterol, and 7.4 to 8.4% by weight cationic detergent was heated for approximately one hour at 86°C. The lipid-oil phase was then blended with an aqueous phase containing water at 65°C using a
20   5 ml syringe machine (as described in U.S. Patent No. 4,895,452, the teachings of which are incorporated by reference herein) on a volume to volume basis of 13 parts lipid-oil to 37 parts water.

25

Example 3 -   Microbicidal Activity of GMO and GMS Oil-in-Water Emulsions Against *Staphylococcus aureus* type 8

In this Example, the microbicidal activities of each of the GMO and GMS emulsions shown in Table 1 were compared. For this purpose, the ability of each of the emulsions to

5   kill *Staphylococcus aureus* type 8 was tested as follows:

Bacteria were innoculated into liquid media (Trypticase soy broth or Schaedler's broth) and incubated at 37°C for 1-7 hours. An optical density at a wavelength of 650 nm and a quantitative culture were then performed on each broth culture. One milliliter of each

10   bacterial broth was then mixed with one milliliter of emulsion, or water as a control, for 10 or 30 minutes. Quantitative cultures were then performed in duplicate on each mixture. Plates containing bacteria were incubated at 37°C for 18 hours and then counted.

The percentage of bacteria killed was then determined by the following equation.

15    $$\% \ kill \ = \ \frac{(A-B)}{A} \ X \ 100$$

A = The total number of bacteria innoculated

B = The total number counted after mixing with an emulsion

20

Table 4 lists the percentages of *Staphylococcus aureus* type 8 killed by each emulsion or water after a 10 or 30 minute incubation with 2×10^7 bacteria.

TABLE 4

Chemical Components of Preparation	% Inactivate After a 10 Minute Incubation	% Inactivate After a 30 Minute Incubation
GMO/CPC	100	100
GMO/CPB	100	100
GMO/CTAB	99.99	99.99
GMO/DDDAB	65	0
GMO/Oleic acid	0	0
GMS/CPC	65	0
Control alone	50	0

25

From Table 4 one can see that the positively charged glycerol monooleate (GMO) emulsions inactivate *Staphylococcus aureus* type 8 while the positively charged glycerol monostearate (GMS/CPC) emulsion fails to inactivate. In addition, only the cationic compounds having a C_{12}-C_{16} chain containing either chloride or bromide, i.e., CPC, CPB or

CTAB, and not the dioctadecyl cationic compound (DDDAB) or oleic acid (having a negative charge), were associated with significant microbicidal activity. The inactivation of this bacterium, *Staphylococcus aureus*, is therefore more effective using GMO emulsions containing a chloride or bromide containing cationic compound having a C_{12}-C_{16} chain.

5

In order to determine the effect of varying the concentration of the charge producing agent in the emulsions of the invention, GMO emulsions containing different concentrations of CPC were also tested for antimicrobicidal activity against *Staphylococcus aureus* type 8. Table 5 shows percentages of *Staphylococcus aureus* type 8 killed after a 10 or 30 minute

10   incubation of the GMO emulsions with 10^7 bacteria.

TABLE 5

Chemical Components of Emulsion	Initial Innoculum of Staphylococcus Aureus Type 8	% Inactivate After a 10 Minute Incubation	% Inactivate After a 30 Minute Incubation
GMO/CPC	10,000,000 CFU [1.8 mg/ml emulsion]	100	100
GMO/CPC	10,000,000 CFU [0.9 mg/ml emulsion]	99.5	100
GMO/CPC	10,000,000 CFU [0.45 mg/ml emulsion]	54	99.5
GMO/CPC	10,000,000 CFU [0.23 mg/ml emulsion]	39	32
GMO/CPC	10,000,000 CFU [0.028 mg/ml emulsion]	0	0
GMO	10,000,000 CFU	0	0
GMO/Oleic acid	10,000,000 CFU	0	0
Water alone	10,000,000 CFU	10	0

Table 5 demonstrates that *Staphylococcus aureus* type 8 bacteria were sensitive to the microbicidal action of the GMO/CPC emulsion at CPC concentrations of greater than 0.23 mg/ml of emulsion.

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Example 4 - Microbicidal Activity of GMO Oil-in-Water Emulsions Against Bacteria and Yeast

In this Example, the ability of the GMO/CPC emulsion formed in Example 1 and shown in Table 1 to kill a variety of bacteria and yeast was tested.

10

The assay described above in Example 3 was used to measure the microbicidal activity of the GMO/CPC emulsion against a number of bacteria and fungi, except for the following changes:

15   Yeast were innoculated into Sabouraud's broth and incubated at 30°C for 6 hours, mixed with the GMO/CPC emulsion for 10 or 30 minutes, plated and incubated at 37 C for 18 hours before counting.

P. acnes was grown anaerobically in Schaedler's broth for 24 hours, mixed with
20   emulsion for 10 or 30 minutes, plated on Trypticase soy agar plates with 5% sheep blood and incubated anaerobically for 72 hours prior to counting.

G. vaginalis was plated on Trypticase soy agar plates with 5% sheep blood and incubated in 5% CO_2 at 37°C for 72 hours. Colonies were swabbed from plates and
25   innoculated into Schaedler's broth at the density of a 0.5 McFarland standard. This broth/bacterial mixture was then incubated with the emulsion for 10 or 30 minutes and then plated on Trypticase soy agar plates with 5% sheep blood. Plates were incubated for 72 hours in 5% CO_2 at 37°C before counting colonies.

30   Table 6 lists the percentage of gram positive bacteria killed after a 10 or 30 minute incubation of the GMO/CPC emulsion with between 10^5 and 10^8 bacteria. The listed bacteria can generally be categorized as follows: (a) those which colonize on the skin which include *Staphylococcus aureus* (type 8), *Staphylococcus aureus* (type 5), *Staphylococcus epidermidis* (strain 977), Group B *Streptococcus* (capsular type III), Group A *Streptococcus*
35   (beta-hemolytic), and *Propionibacterium acneus*; (b) those which colonize in the oropharynx which include Group A *Streptococcus* (beta-hemolytic), *Streptococcus pneumoniae* (type 5), and *Streptococcus mutans*; and (c) those which colonize or infect the vagina which include *Gardnerella vaginalis* and Group B *Streptococcus* (capsular type III).

12

TABLE 6

Gram Positive Bacteria	Innoculum (CFU)•	% Inactivate after a 10 Minute Incubation	% Inactivate after a 30 Minute Incubation
Staphylococcus aureus (type 8/bactermic isolate)	2×10^7	99.99	99.99
Staphylococcus aureus (type 5 bacteremic isolate)	9×10^6	100	99.99
Staphylococcus epidermidis (strain 977)	8×10^5	100	100
Group B *Streptococcus* (capsular type III)	2.9×10^7	99.99	100
Group A beta-hemolytic (type 1) *Streptococcus* (ATCC 12344)	3.3×10^7	99.99	99.99
Listeria monocytogenes (type 2) (ATCC 19112)	1.3×10^8	99.99	99.99
Streptococcus pneumoniae (type 5) (ATCC 6305)	6.4×10^7	100	100
Streptococcus mutans (ATCC 25179)	6.5×10^6	96.2	96.8
Propionibacterium acnes (ATCC 6919)	1.2×10^8	100	100
Enterococcus fecalis (ATCC 19433)	3.7×10^7	99.36	99.98
Gardnerella vaginalis (ATCC 14018)	5.5×10^7	100	100
Lactobacillus acidophilus (ATCC 4356)	5.0×10^5	100	100

• CFU = colony forming units

Table 6 demonstrates that all gram positive bacteria which cause significant human
clinical infections were exquisitely sensitive to the microbicidal action of the GMO/CPC
emulsion.

Table 7 shows the percentage of gram negative bacteria killed after a 10 or 30 minute
incubation of the GMO/CPC emulsion with between 10^5 and 10^8 bacteria. Included in the
table are gram negative bacteria which colonize in the oropharynx, such as *Haemophilus*

influenzae (capsular type b) and *Neisseria meningitidis* type b; the gastrointestinal tract, such as *Helicobacter pylori*; and the vagina, such as *Neisseria gonorrhoeae*.

TABLE 7

Gram Negative Bacteria	Innoculum (CFU)•	% Inactivate after a 10 Minute Incubation	% Inactivate after a 30 Minute Incubation
Escherichia coli type O18:K1	2.1×10^7	97.1	96.7
Escherichia coli type O18:K-	3.2×10^7	89.7	99.6
Escherichia coli J5 (epimerase deficient)	3×10^7	94	85
Flavobacterium meningosepticum Group A (ATCC 13253)	2.1×10^6	0	47.6
Klebsiella pneumonia type O12K+	5×10^7	98.3	99.9
Pseudomonas aeruginosa type FD-1	3.8×10^7	99	99
Pseudomonas aeruginosa MEP strain 2192	8×10^6	99.1	97.5
Haemophilus influenzae capsular type b (ATCVC 33533)	1×10^7	99.99	99.99
Neisseria meningitidis type b (ATCC 13090)	1.6×10^8	100	100
Neisseria gonorrhoeae (ATCC 9793)	1.2×10^6	100	100
Helicobacter pylori (ATCC 43504)	3×10^6	100	100
Helicobacter pylori (ATCC 43629)	4.7×10^6	100	100
Helicobacter pylori (ATCC 49503)	2×10^6	100	100
Helicobacter pylori (ATCC 43579)	7×10^6	100	100

5

Table 7 illustrates that at 30 minutes there is inactivation of at least 85% of the innoculum of all gram negative bacteria tested except for *Flavobacterium meningosepticum*. The encapsulated type b *Haemophilus influenzae*, type b *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Helicobacter pylori* bacteria, which have relatively rough LPS types

10   compared to the other gram negative bacteria, are exquisitely sensitive to the microbicidal activity of the GMO/CPC emulsion.

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Table 8 shows the percentage of the two *Candida* species killed after a 10 or 30 minute incubation of the GMO/CPC emulsion with 10^5 yeast.

TABLE 8

Yeast	Innoculum (CFU)	% Inactivate After a 10 Minute Incubation	% Inactivate After a 30 Minute Incubation
Candida albicans (clinical blood isolate)	3.2×10^5	62.5	62.5
Candida tropicalis (clinical blood isolate)	5.4×10^5	100	100

5

Table 8 demonstrates that significant inactivating of the *Candida albicans* species occurs after only a 10-30 minute incubation with the GMO/CPC emulsion.

10   **Example 5 -   Microbicidal Activity of 10N GMO Emulsions**

In this Example, the positively charged 10N GMO emulsions formed in Example 2 and shown in Table 3 were tested for microbicidal activity against two gram positive bacteria, *Staphylococcus aureus* type 8 and *Staphylococcus epidermidis*, and two gram negative bacteria, *E. coli* and *Helicobacter pylori*, as follows:

15

To lyophilized cultures of bacteria, 0.5 mL of Schaedler's Broth was added and the entire contents of the vial inoculated to a 5% Sheep blood agar plate (TSA ll). Cultures were then incubated in an anaerobic jar with Campy GasPAK at 37°C for 72 hours. Colonies from 72 hour TSA ll plates were then innoculated into Schaedler's broth until an 0.5 McFarland
20   Standard was achieved.

A one ml aliquot of the bacterial culture was then mixed with one ml of oil-in-water emulsion for 10 minutes. Quantitative cultures were then performed in duplicate on each mixture, and a quantitative culture was performed on the original broth culture. Plates
25   containing bacteria were incubated at 37°C for 72 hours in an anaerobic jar with Campy GasPaks and then counted. The percentage of bacteria killed were determined by the following equation and the results are shown in Table 9:

$$\% \text{ Kill} = \frac{(A-B)}{A} \times 100$$

30

15

A = The total number of bacteria innoculated

B = The number counted after mixing with an emulsion or liposomes

Table 9 lists the percentages of each bacterium killed after a 10 minute incubation with the 10N GMO/CPC emulsion from Table 3 with between 10^6 and 10^7 bacteria. The 10N GMO/CPC emulsion was tested in undiluted form and in several dilutions, as indicated in Table 9.

TABLE 9

Bacterium	Dilution	Trial # 1	Trial # 2
Staphylococcus aureus	Undilute	NT	100.00%
Type 8	1 : 10		99.99%
8 x 10E7 CFU/mL	1 : 20		99.99%
10 minute incubation	1 : 40		99.99%
	1 : 80		96.25%
Staphylococcus	Undilute	NT	100.00%
epidermidis 977	1 : 10		100.00%
9 X 10E6 CFU/mL	1 : 20		100.00%
10 minute incubation	1 : 40		99.98%
	1 : 80		99.97%
Escherichia coli	Undilute	NT	99.99%
Type 02a,2b	1 : 10		99.99%
1 X 10E7 CFU/mL	1 : 20		99.98%
10 minute incubation	1 : 40		94.00%
	1 : 80		50.00%
Helicobacter pylori 43579	Undilute	100.00%	100.00%
10 minute incubation	1 : 10	100.00%	100.00%
Inoculum for Trial #1 5X10E6 CFU/ml	1 : 20	100.00%	100.00%
Inoculum for Trial #2 2X10E7 CFU/ml	1 : 40	99.00%	99.00%
	1 : 80	99.20%	99.00%

NT = Not Tested on This Date

Table 9 shows that at 10 minutes there is a greater than 96% inactivation of each bacterium (except for *E. coli* at high dilutions (i.e., 1 : 40 and 1 : 80)) tested with the 10N

16

GMO/CPC emulsion up to a 1 : 80 dilution. At a dilution of 1 : 10, there is a 99.99-100% inactivation of each bacterium.

5   **Example 6 -   Viricidal Activity of GMO Oil-in-Water Emulsions and Fusogenic Lipid Vesicles Against HIV**

In this Example, the viricidal activity of the GMO/CPC emulsion formed in Example 1 and fusogenic lipid vesicles prepared as described below were tested against HIV.

10   To prepare fusogenic lipid vesicles, 94.5 grams of Polyoxyethylene (POE) 2 Stearyl Ether (Brij 72), 0.78 grams of oleic acid, and 36 grams of cholesterol were heated to melting to form a liquid lipid phase. A portion of that lipid phase, 0.53 ml, was then mixed with 0.47 ml of squalene to form 1.0 ml of a lipid-oil phase. This was blended with an aqueous phase containing 4.0 ml of PBS under shear mixing conditions to form the lipid vesicles, as
15   described in U.S. Patent 4,911,928. "Shear mixing conditions", as used herein, means a shear equivalent to a relative flow of 5-50 m/s through a 1mm orifice. On a volume to volume basis, the mixture contained 4 parts diluent to 1 part lipid-oil mixture, or 11.7% lipid to 8.3% oil to 80% diluent.

20   The GMO/CPC emulsion and Brij 72 fusogenic lipid vesicles were assayed for viricidal activity against HIV as follows:

A cell suspension containing 20 x 10^6 C8166 cells, a HTLV-1 transformed T cell line, in 20 ml of RPMI media containing 10% PBS and 50 μg/ml gentamicin was prepared
25   and divided into 0.4 ml aliquots. A series of viral dilutions (10^{-2} to 10^{-6}) were then made by first mixing 100 microliters of HIV-1$_{Mn}$ 1000 x pelleted virus with either 100 microliters of sterile water or 100 microliters of the GMO/CPC emulsion for 30 minutes at room temperature on a nutator. 1.8 ml of tissue culture medium was then added to each preparation, resulting in a dilution of 10^{-1}. Serial 10-fold dilutions were then performed from
30   10^{-2} to 10^{-6} by taking 150 microliters of the 10^{-1} dilution and serially diluting it out in 1.35 ml of RPMI media five more times.

Two 0.4 ml aliquots of each dilution were then added to the aliquots of C8166 cells, prepared as described above. The samples were incubated at 37°C for either 2, 6 or 24 hours,
35   washed 3 times in phosphate buffered saline, resuspended in fresh medium and each dilution plated in four replicate wells. Cells were fed twice weekly. Cells were observed at 7 and 14 days for cytopathic effects and supernatants were collected to measure levels of p24 as an indication of viral proliferation. Tissue culture infectious dose (TCID-50 (log_{10})) calculations were then performed based on the measured levels of p24. The results were as follows:

For samples incubated for 2 hours with the GMO/CPC emulsion, there was a 4.5 log
virus reduction, as compared with water, in the TCID-50 at 7 days and a 4.25 log virus
reduction in the TCID-50 at 14 days.

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For samples incubated for 6 hours with the GMO/CPC emulsion, there was a 4.5 log
virus reduction, as compared with water, in the TCID-50 at 7 days and a 5 log virus
reduction, as compared with water, in the TCID-50 at 14 days.

10   For samples incubated for 24 hours with the GMO/CPC emulsion, there was a 4.0 log
virus reduction, as compared with water, in the TCID-50 at 7 and 14 days; there was a 3.75
and 4.25 log reduction, as compared with Phosphate Buffered Saline, in the TCID-50 at 7 and
14 days, respectively; and there was a 4.0 and 4.5 log reduction, as compared with fusogenic
novasomes containing POE 2 Stearyl Ether, in the TCID-50 at 7 and 14 days, respectively.

15

Overall, this Example demonstrates that GMO/CPC emulsions inactivate 4.0 to 5.0
logs of HIV-1_{Mn} infectivity in C8166 tissue culture assays. These experiments, which can be
performed using any enveloped virus, demonstrate the effectiveness of these materials to
inactivate viruses such as HIV-1_{Mn}.

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Example 7 - Stability of GMO Oil-in-Water Emulsions

In this Example, the GMO/CPC emulsion formed in Example 1 was tested for
stability in the presence of heat, acid and base. Table 10 shows the effect of boiling for one
25   hour on breakdown or aggregation of the GMO/CPC emulsion, shows the effects of mixing
equal volumes of 1N Nitric acid and GMO/CPC emulsion for two hours on breakdown or
aggregation of the GMO/CPC emulsion, and the effects of mixing equal volumes of 1N
Sodium hydroxide and GMO/CPC emulsion for two hours on breakdown or aggregation of
the GMO/CPC emulsion.

TABLE 10

Chemical Components of Emulsion	Intervention	Mean Coulter Size in Microns	Mean Coulter Range in Microns
GMO/CPC	No boiling	1.008	0.720-1.337
GMO/CPC	Boiling 1 hour	1.167	0.654-1.517
GMO/CPC	No acid treatment	1.008	0.720-1.337
GMO/CPC	1N HNO$_3$ for 2 hours	1.062	0.675-1.569
GMO/CPC	No base treatment	1.008	0.720-1.337
GMO/CPC	1N NaOH for 2 hours	0.804	0.658-0.969

Table 10 shows that : (a) boiling for 1 hour does not significantly alter the breakdown or size of the emulsion; (b) 1N Nitric acid exposure for 2 hours does not significantly alter the size or aggregate profile of the CMO/CPC emulsion; and (c) 1N Sodium hydroxide exposure for 2 hours causes a 20% decrease in the mean size of the emulsion without disrupting the emulsion or causing aggregation.

From the above-described Examples 1-7, it is evident that the antimicrobial oil-in-water emulsions of the present invention have significant microbicidal activity against a wide variety of viruses, bacteria and yeast, even at extremely low concentrations of cationic halogen-containing compound, such as CPC. Furthermore, the emulsions of the invention are stable in the presence of heat, acid, and base, making them very suitable for pharmaceutical administration, whether topical, oral or systemic.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1.   An antimicrobial oil-in-water emulsion comprising positively charged droplets of an oily discontinuous phase dispersed in a continuous aqueous phase, the emulsion comprising:

      a.   an oil;

      b.   a glycerol ester; and

      c.   a cationic halogen-containing compound having a C_{12}-C_{16} chain.

2.   An antimicrobial emulsion of claim 1, wherein the glycerol ester is selected from the group consisting of glycerol monooleate, glycerol trioleate, glycerol monolaurate, and glycerol dilaurate.

3.   An antimicrobial emulsion of claim 1, wherein the oil is selected from the group consisting of soybean oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, avocado oil, flavor oils, water insoluble vitamins, and mixtures thereof.

4.   An antimicrobial emulsion of claim 1, wherein the halogen is selected from the group consisting of bromine, chlorine, and fluorine.

5.   An antimicrobial emulsion of claim 1, wherein the cationic halogen-containing compound having a C_{12}-C_{16} chain is selected from the group consisting of cetylpyridinium chloride, cetylpyridinium bromide, cetyltrimethylammonium bromide, cetyltrimethylammonium chloride, cetyldimethylethylammonium bromide, cetylbenzyldimethylammonium chloride, cetyltributylphosphonium bromide, dodecyltrimethylammonium bromide, and tetradecyltrimethylammonium bromide.

6.   An antimicrobial emulsion of claim 1, wherein the emulsion further comprises at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

7.   The antimicrobial emulsion of claim 1, wherein at least a portion of the emulsion is in the form of a positively charged lipid structure selected from the group consisting of unilamellar, multilamellar, and paucilamellar lipid vesicles, micelles, and lamellar phases.

8.   An antimicrobial preparation suitable for pharmaceutical administration comprising the antimicrobial emulsion of claim 1 and a pharmaceutically acceptable carrier.

20

     9.   An antimicrobial oil-in-water emulsion comprising positively charged droplets of an oily discontinuous phase dispersed in a continuous aqueous phase, the emulsion comprising:

          a.   an oil selected from the group consisting of soybean oil, squalene oil, squalane oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, avocado oil, flavor oils, water insoluble vitamins, and mixtures thereof;

          b.   glycerol monooleate; and

          c.   cetylpyridinium chloride.

     10.   An antimicrobial preparation suitable for pharmaceutical administration comprising the antimicrobial emulsion of claim 9 and a pharmaceutically acceptable carrier.

     11.   A method of inhibiting the growth of an infectious pathogen comprising the step of topical application of an antimicrobial oil-in-water emulsion,
said antimicrobial emulsion being in the form of positively charged droplets of an oily discontinuous phase dispersed in a continuous aqueous phase, the emulsion comprising:

          a.   an oil;

          b.   a glycerol ester; and

          c.   a cationic halogen-containing compound having a C_{12}-C_{16} chain.

     12.   The method of claim 11, wherein the glycerol ester is selected from the group consisting of glycerol monooleate, glycerol trioleate, glycerol monolaurate, and glycerol dilaurate as the primary emulsifier.

     13.   The method of claim 11, wherein the halogen is selected from the group consisting of bromine, chlorine, and fluorine.

     14.   The method of claim 11, wherein the cationic halogen-containing compound having a C_{12}-C_{16} chain is selected from the group consisting of cetylpyridinium chloride, cetylpyridinium bromide, cetyltrimethylammonium bromide, cetyldimethyethylammonium bromide, and benzalkonium chloride.

     15.   The method of claim 11, wherein the oil is selected from the group consisting of soybean oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, avocado oil, flavor oils, water insoluble vitamins, and mixtures thereof.

16.   The method of claim 11, wherein the emulsion further comprises at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

5   17.   The method of claim 11, wherein said topical application comprises oral application.

18.   The method of claim 11, wherein said topical application comprises application to a mucous membrane via inhalation.

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19.   The method of claim 11, wherein said topical application comprises vaginal application.

20.   The method of claim 11, wherein the infectious pathogen is a bacterium.

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21.   The method of claim 11, wherein the infectious pathogen is a fungus.

22.   The method of claim 11, wherein the infectious pathogen is a virus.

20   23.   The method of claim 22, wherein the virus is an enveloped virus.

24.   The method of claim 23, wherein the enveloped virus is HIV.

25.   The method of claim 23, wherein the enveloped virus is selected from the

25   group consisting of *Herpesviridae, Togaviridae, Flaviviridae, Coronaviridae, Rhabdoviridae, Filoviridae, Paramyxoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Retroviridae,* and *Hepadnavirida.*

26.   A method of inhibiting the growth of an infectious pathogen comprising the

30   step of topical application of an antimicrobial oil-in-water emulsion to an area containing the infectious pathogen, the antimicrobial emulsion being in the form of positively charged droplets of an oily discontinuous phase dispersed in a continuous aqueous phase, the emulsion comprising:

a.   an oil selected from the group consisting of soybean oil, squalane oil,

35   sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, avocado oil, flavor oils, water insoluble vitamins, and mixtures thereof;

b.   glycerol monooleate; and

c.   cetylpyridinium chloride.

22

27.   A method of inhibiting the growth of an infectious pathogen *in vivo* comprising the step of systemically administering to a subject an antimicrobial oil-in-water emulsion, said antimicrobial emulsion being in the form of positively charged droplets of an oily discontinuous phase dispersed in a continuous aqueous phase, the emulsion comprising:

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a.   an oil selected from the group consisting of soybean oil, squalene oil, sesame oil, olive oil, canola oil, corn oil, rapeseed oil, safflower oil, sunflower oil, fish oils, avocado oil, flavor oils, water insoluble vitamins, and mixtures thereof;

b.   a glycerol ester selected from the group consisting of glycerol

10   monooleate, glycerol trioleate, glycerol monolaurate, and glycerol dilaurate; and

c.   a cationic halogen-containing compound having a C_{12}-C_{16} chain selected from the group consisting of cetylpyridinium chloride, cetylpyridinium bromide, cetyltrimethylammonium bromide, cetyldimethyethylammonium bromide, and benzalkonium chloride.

15

28.   The method of claim 27, wherein the emulsion further comprises at least one sterol selected from the group consisting of cholesterol, cholesterol derivatives, hydrocortisone, phytosterol, and mixtures thereof.

20   29.   The method of claim 27 wherein said step of systemically administering comprises enteral administration.

30.   The method of claim 27, wherein the pathogen is selected from the group consisting of bacteria, fungi, and viruses.

25

31.   A method of treating an infection by a pathogen in a subject comprising the step of systemically administering to the subject an antimicrobial oil-in-water emulsion comprising positively charged droplets of an oily discontinuous phase dispersed in a continuous aqueous phase, the emulsion comprising:

30   a.   an oil;

b.   glycerol monooleate; and

c.   cetylpyridinium chloride.

32.   A method of inactivating an infectious pathogen in vitro comprising the step

35   of contacting the pathogen with an antimicrobial oil-in-water emulsion, the emulsion being in the form of positively charged droplets of an oily discontinuous phase dispersed in a continuous aqueous phase, the emulsion comprising:

a.   an oil selected from the group consisting of soybean oil, squalane oil, sesame oil, olive oil, canola oil, corn oil, rapeseed, safflower oil, sunflower oil, fish oils,

petrolatum, avocado oil, triglyceride oils and fats, flavor oils, water insoluble vitamins, and mixtures thereof;

    b.   a glycerol ester selected from the group consisting of glycerol monooleate, glycerol trioleate, glycerol monolaurate, and glycerol dilaurate; and

    c.   a cationic halogen-containing compound having a C_{12}-C_{16} chain selected from the group consisting of cetylpyridinium chloride, cetylpyridinium bromide, and cetyltrimethylammonium bromide.

33.   The method of claim 32, wherein the pathogen is a virus.

34.   The method of claim 33, wherein the virus is HIV.

INTERNATIONAL SEARCH REPORT

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)   : A61K/06
US CL   : 424/401

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   424/401

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN-CAS; APS

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US,A, 5,362,494 (Zysman et al) 08 November 1994, column 7-11.	1-34

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

Date of the actual completion of the international search	Date of mailing of the international search report
12 JULY 1995	28 JUL 1995

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(54) Title: TARGETED HETERO-ASSOCIATION OF RECOMBINANT PROTEINS TO MULTI-FUNCTIONAL COMPLEXES

(57) Abstract

The present invention relates to a method for targeted assembly of distinct active peptide or protein domains into a single complex and to such complexes. The invention relates particularly to the fusion of peptide or protein domains to complementary association domains which are derived from a single tertiary or quaternary structure by segmentation. The association domains are designed to assemble in a complementary fashion, thereby providing multifunctional (poly)peptides.

TARGETED HETERO-ASSOCIATION OF RECOMBINANT PROTEINS TO MULTI-FUNCTIONAL COMPLEXES

Background of the Invention

Increasingly, there is a need for proteins which combine two or more functions, such as binding or catalysis, in a single structure. Typically, proteins which combine two or more functions are prepared either as fusion proteins or through chemical conjugation of the component functional domains. Both of these approaches suffer from disadvantages. Genetic "single chain" fusions suffer the disadvantages that (i) only a few (2-3) proteins can be fused (Rock et al., 1992, *Prot. Eng. 5*, 583-591), (ii) mutual interference between the component domains may hinder folding, and (iii) the size of the fusion protein may make it difficult to prepare. The alternative, chemical cross-linking *in vitro* following purification of independently expressed proteins, is difficult to control and invariably leads to undefined products and to a severe loss in yield of functional material.

Recently, methods for achieving non-covalent association of two or more of the same functional domains have been developed. This can be achieved through the use of domains attached to peptides which self-associate to form homo-multimers (Pack & Plückthun, 1992, *Biochemistry 31*, 1579-1584). For example, the association of two separately expressed scFv antibody fragments by C-terminally fused amphipathic helices *in vivo* provides homo-dimers of antibody fragments in *E. coli* (PCT/EP93/00082; Pack et al., 1993, *Bio/Technology 11*, 1271-1277) or homo-tetramers;(Pack et al., 1995, *J. Mol. Biol., 246*, 28-34).

To assemble distinct protein functions such as two antibody fragments with different specificities fused to such association domains, the helices must have a tendency to form hetero-multimers. In principle, this could be achieved with complementary helices such as the hetero-dimerizing JUN and FOS zippers of the AP-1 transcription factor (O'Shea et al., 1992, *Cell 68*, 699-708). The clear disadvantage of association domains based on hetero-associating helices,

2

however, is their pseudo-symmetry and their similar periodicity of hydrophobic and hydrophilic residues. This structural similarity results in a strong tendency to form homo-dimers and, thus, to lower significantly the yield of hetero-dimers (O'Shea et al., 1992, *Cell 68*, 699-708; Pack, 1994, Ph. D. thesis, Ludwig-Maximilians-Universität München). Furthermore, the formation of JUN/FOS hetero-dimers is kinetically disfavoured and requires a temperature-dependent unfolding of the kinetically favoured homo-dimers, especially JUN/JUN homo-dimers (PCT/EP93/00082; O'Shea et al., 1992, *Cell 68*, 699-708; Pack, 1994, Ph. D. thesis, Ludwig-Maximilians-Universität München). Because of the need for additional purification steps to separate the unwanted homo-dimers from hetero-dimers and the resulting decrease in yield, hetero-association domains based on amphipathic helices do not result in practical advantages compared to conventional chemical coupling.

These disadvantages of the prior art are overcome by the present invention which provides multi-functional polypeptides and methods for the preparation of these multi-functional proteins. This is achieved via the use of association domains which are designed to associate predominantly in a complementary fashion, and not to self-associate.

Detailed Description of the Invention

In the earliest steps of protein folding, peptide chains form a disordered hydrophobic core by collapsing hydrophobic residues into the interior of an intermediate "molten globule". This hydrophobic effect is considered to be the most important driving force of folding (Matthews, 1993, *Annu. Rev. Biochem. 62*, 653 - 683; Fersht, 1993, *FEBS Letters 325*, 5 - 16). The burial of hydrophobic residues and the resulting exclusion of solvent is the determining factor in the stability of compact tertiary structures such as acyl-phosphatase (Pastore et al, J. Mol. Biol. 224, 427-440, 1992) interleukin-2 (Brandhuber et al., 1987, *Science 238*, 1707 - 1709), calbindin (Parmentier, 1990, *Adv. Exp. Med. Biol. 269*, 27-34) or ubiquitin (Briggs & Roder, 1992, *Proc. Natl. Acad. Sci. USA 89*, 2017 - 2021).

This concept forms the basis of the present invention, which provides individually encoded peptides or "segments" which, in a single continuous chain, would comprise a compact tertiary structure with a highly hydrophobic core. The component peptides are chosen so as to be asymmetric in their assumed structure, so as not to self-associate to form homo-multimers, but rather to associate in a complementary fashion, adopting a stable complex which resembles the parent tertiary structure. On the genetic level, these segments are encoded by interchangeable cassettes with suitable restriction sites. These standardized cassettes are fused C- or N-terminally to different recombinant proteins via a linker or hinge in a suitable expression vector system.

Thus, the present invention relates to a multi-functional polypeptide comprising:

(a)   a first amino acid sequence attached to at least one functional domain;

(b)   a second amino acid sequence attached to at least one further functional domain; and

(c)   optionally, further amino acid sequences each attached to at least one further functional domain;

wherein any one or more of said amino acid sequences interacts with at least one of said amino acid sequences in a complementary fashion to form a parental, native-like tertiary or optionally quaternary structure and wherein the parental, native-like tertiary or optionally quaternary structure is derived from a single parent polypeptide. In this context, the term parent polypeptide refers to a polypeptide which has a compact tertiary or quarternary structure with a hydrophobic core. The invention provides for many different parent polypeptides to be used as the basis for the association domain. Suitable polypeptides can be identified by searching for compact, single-domain proteins or protein fragments in the database of known protein structures (Protein Data Bank, PDB) and selecting structures that are stable and can be expressed at high yields in recombinant form. These structures can then be analyzed for hydrophobic sub-clusters by the method of Karpeisky and Ilyn (1992, *J. Mol. Biol.* *224*, 629-638) or for structural units (such as ß-elements or helical hairpin structures) by standard molecular modelling techniques. In a further embodiment, the present invention

4

provides for multi-functional polypeptides wherein the single parent polypeptide is taken from the list ubiquitin, acyl phosphatase, IL-2, calbindin and myoglobin.

In a preferred embodiment, the present invention provides a multi-functional polypeptide comprising two or more amino acid sequences each attached to at least one functional domain, wherein any two or more of said amino acid sequences can associate in a complementary fashion to provide a parental, native like, tertiary or optionally quaternary structure.

Once structural sub-domains are identified, the protein is dissected in such a way these sub-domains remain intact. The selection process can be expanded to proteins for which no structure is available but which satisfy the criteria of stability and good expression. For these proteins, folding sub-domains can be determined by hydrogen exchange pulse-labelling of backbone amides during the folding reaction, followed by NMR detection in the native state (Roder et al., 1988, *Nature 355*, 700-704; Udgaonkar & Baldwin, 1988, *Science 255*, 594-597). Alternatively, folding sub-domains can be identified by mild proteolysis, denaturation, purification of fragments and reconstitution *in vitro* (Tasayco & Carey, 1992, *Science 255*, 594-597; Wu et al., 1993, *Biochemistry 32*, 10271-10276). Finally, additional clues for the choice of cleavage sites can be obtained from the exon structure in the case of eukaryotic proteins, since the exons frequently (though not always) correspond to structural sub-domains of a protein. This has, for example, been discussed for the case of myoglobin (Go 1981, *Nature 291*, 90).

The yield of properly assembled molecules is expected to decrease significantly for constructs in which a protein domain is divided into three or more parts. This is due to the fact that several sub-domains must come together simultaneously to form a viable structure. This effect is countered by dividing the polypeptide chain into sub-domains that represent folding units (identified by the methods described above). Thus, not only the final, assembled complex but also assembly intermediates will have the stability necessary to allow their accumulation in the

5

host during expression, resulting in a greatly improved kinetic behaviour of the system.

In solution, the isolated segments have little secondary structure and remain monomeric or form transient, non-specific and easily disrupted aggregates. Only upon mixing, either by separate expression and purification, or by co-expression, can the concerted folding of complementary segments provide the necessary intermediate interaction of residues (Matthews, 1993, Annu. Rev. Biochem. 62, 653 - 683) that results in the formation of a compact, native-like structure. This association, mainly driven by the burial of hydrophobic residues of all segments into a single hydrophobic core, leads to a targeted assembly of the N- or C-terminally fused proteins to a multi-functional complex in vivo or in vitro.

Optionally, the reconstituted native-like structure may also contribute an enzymatic or binding activity to increase the number of effector functions in the assembled complex. Accordingly, the present invention also provides a multi-functional polypeptide as described above, in which the native-like, tertiary or quaternary structure provides a biological activity. For example, when acyl phosphatase is used as the basis of the association domain, it is expected that the multi-functional polypeptide will retain some phosphatase activity.

The present invention provides for many different types of functional domains to be linked into the multi-functional polypeptide. Particularly preferred are cases in which one or more, preferably two, of said functional domains are fragments derived from molecules of the immunoglobulin superfamily. In particularly preferred embodiments, said fragments are antibody fragments. Also preferred are cases in which at least one of the functional domains possesses biological activity other than that associated with a fragment derived from a member of the immunoglobulin superfamily. By way of example, the present invention provides for the targeted assembly of enzymes, toxins, cytokines, peptide hormones, immunoglobulins, metal binding domains, soluble receptors, lectins, lipoproteins, purification tails and bioactive peptides to multi-functional complexes (Fig. 1) based on a modular system of expression vectors, restriction sites and "plug-in" gene cassettes coding for assembly segments, peptide linkers and functional domains (Fig.2).

6

If covalent linkage between the segments is necessary to prevent dissociation at low concentrations, cysteines can be introduced to form inter-segmental disulphide bridges between the amino acid sequences which comprise the association domain (Ecker et al., 1989, *J. Biol. Chem.* 264, 1887-1893; Pack & Plückthun, 1992, *Biochemistry 31*, 1579-1584). Accordingly, the present invention provides multi-functional polypeptides wherein the folding of the component amino acid sequences is stabilized by a covalent bond.

In order to provide some flexibility between the association domain and the appended functional domains, it may be desired to incorporate a linker peptide. Accordingly, the present invention provides for multi-functional polypeptides of the type described above wherein at least one of the functional domains is coupled to said amino acid sequence via a flexible peptide linker. By way of example, the flexible linker may be derived from the hinge region of an antibody.

The invention enables even more complex multi-functional polypeptides to be constructed via the attachment of at least one further (poly)peptide to one or more of said amino acid sequences. By way of example, the further (poly)peptide can be taken from the list enzymes, toxins, cytokines, peptide hormones, immunoglobulins, metal binding domains, soluble receptors, lectins, lipoproteins, purification tails, in particular peptides which are able to bind to an independent binding entity, bioactive peptides, preferably of 5 to 15 amino acid residues, metal binding proteins, DNA binding domains, transcription factors and growth factors.

For therapeutic purposes, it is often desirable that proteinaceous substances display the minimum possible immunogenicity. Accordingly, the present invention provides for multi-functional polypeptides as described above in which at least one of said amino acid sequences, functional domains, or further (poly)peptides is of human origin.

In addition to the peptides and proteins provided above, the present invention also provides for DNA sequences, vectors, preferably bicistronic vectors, vector cassettes, characterised in that they comprise a DNA sequence encoding an amino acid sequence and optionally at least one further (poly)peptide comprised in the multifunctional polypeptide of the invention, and additionally at least one,

preferably singular cloning sites for inserting the DNA encoding at least one further functional domain or that they comprise DNA sequences encoding the amino acid sequences, and optionally the further (poly)peptide(s) comprised in the multifunctional polypeptide of the invention and suitable restriction sites for the cloning of DNA sequences encoding the functional domains, such that upon expression of the DNA sequences after the insertion of the DNA sequences encoding the functional domains into said restriction sites, in a suitable host the multifunctional polypeptide of the invention is formed. In a preferred embodiment said vector cassette is characterised in that it comprises the inserted DNA sequence(s) encoding said functional domain(s) and host cells transformed with at least one vector or vector cassette of the invention which can be used for the preparation of said multi-functional polypeptides.

In a further preferred embodiment, said host cell is a mammalian, preferably human, yeast, insect, plant or bacterial, preferably E. coli cell.

The invention further provides for a method for the production of a multifunctional polypeptide of the invention, which comprises culturing the host cell of the invention in a suitable medium, and recovering said multifunctional polypeptide produced by said host cell.

In a further embodiment, the invention relates to a method for the production of a multifunctional polypeptide of the invention which comprises culturing at least two host cells of the invention in a suitable medium, said host cells each producing only one of said first and said second amino acid sequences attached to at least one further functional domain, recovering the amino acid sequences, mixing thereof under mildly denaturing conditions and allowing in vitro folding of the multifunctional polypeptide of the invention from said amino acid sequences.

In a particular preferred embodiment, said method is characterised in that the further amino acid sequences attached to at least one further functional domain

8

are/is produced by at least one further host cell not producing said first or second amino acid sequence.

In another particularly preferred embodiment of the invention, said method is characterised in that at least one further amino acid sequence attached to at least one further functional domain is produced by the host cell of the invention producing said first or second amino acid sequence.

In further preferred embodiments, the present invention provides for pharmaceutical and diagnostic compositions comprising the multi-functional polypeptides described above, said pharmaceutical compositions optionally comprising a pharmaceutically acceptable carrier. Finally, the invention provides for a kit comprising one or more vector cassettes useful in the preparation of said multi-functional polypeptides.

The invention is now illustrated by reference to the following examples, which are provided for the purposes of illustration only and are not intended to limit the scope of the invention.

Example 1: Segmented human ubiquitin as an assembly device

Ubiquitin is a compact intracellular protein of only 76 residues (Fig. 3) and a molecular weight of 5 kDa. It shows the highest conservation among all known proteins and is involved in the degradation pathway of intracellular eukaryotic proteins by forming intermediate isopeptide bonds to its C-terminus and to Lys48 (Hershko & Ciechanover, 1992, *Ann. Rev. Biochem. 61*, 761-807).

To use ubiquitin as an assembly device, the unwanted function can be abolished by truncation of the last three C-terminal residues (--Arg-Gly-Gly), and the exchange of Lys48 to Arg, which prevents the formation of isopeptide bonds to this residue. The altered sequence is then divided in a loop at position Gly36, so that the hydrophobic core falls apart into two segments (called ALPHA and BETA). The synthetic nucleotide sequence of the segments (Fig 4, 5) carry appropriate restriction sites (MroI-HindIII) at the termini, so that the cassette encoding the segments can be easily ligated to a EcoRI-MroI cassette encoding the flexible linker (hinge of huIgG3; Fig. 6). The cassettes are inserted into the expression vector pIG3 (EcoRI-HindIII; Fig. 7) encoding the scFv fragment of the antibody McPC603 under the lac promoter/operator (Ge et al., 1995, in: *Antibody engineering: A practical approach*. IRL Press, New York, Borrebaeck ed., 229-261). Insertion of a second functional fragment (scFv fragment of the anti-ß-lactam antibody 2H10 with phoA signal sequence) linked to association segment BETA as an XbaI-HindIII DNA fragment (Fig. 8) results in a di-cistronic expression vector (Pack, 1994, Ph. D. thesis, Ludwig-Maximilians-Universität München). After induction with IPTG and translation, the signal sequences guide the antibody fragments fused to the assembly segments to the periplasm, where they assemble to a complex with a reconstituted native-like ubiquitin fold and two different antibody specificities. The complex, a bispecific immunoglobulin, can be recovered and purified by affinity chromatography of cell extract (Pack, 1994, Ph. D. thesis, Ludwig-Maximilians-Universität München).

Example 2: Covalent linkage of the native-like tertiary structure of the assembly device by engineered disulphide bridges and combination of a C-terminal peptide linker with an in-frame restriction site.

The conformational stability of undivided, native ubiquitin can be enhanced by introduction of disulfides at positions 4 and 66 without perturbation in the backbone (Ecker et al., 1989, *J. Biol. Chem. 264*, 1887-1893; Fig. 9). In the context of this invention, the engineering of disulfide bridges provides the covalent linkage of segments (Fig. 10, 11) after co-folding and assembly.

To raise the number of possible functional domains in the assembled complex, a C-terminal peptide can be fused to one or more of the segments of the assembly device. To fuse a functional domain like an enzyme, cytokine, antibody fragment, purification peptide or toxin to this linker, a restriction site, preferably unique, has to be introduced in-frame (Fig. 11). Gene synthesis, cloning, expression as well as recovery of the assembled, covalently linked complex is according to example 1.

Example 3: Segmented human interleukin-2 (IL2) as an assembly device

Human interleukin-2 (Brandhuber et al., 1987, *Science 238*, 1707 - 1709; Kuziel & Greene, 1991, in: *The Cytokine Handbook. Academic Press*, 84-100) is used as an assembly device by segmentation between position His79 and Lys 80 (Fig. 12). The device, encoded by Mrol-Ascl-Hindll gene cassettes (Fig. 13, 14) combines the low immunogenicity of the plasmatic protein with a preferable effector function of the native-like cytokine structure and an inter-segmental cysteine bridge (Cys58-Cys105) after assembly. The combination of one or more antibody fragments against tumor antigens with additional cytokines like IL6 or

IL12 targets the multi-cytokine complex (Rock et al., 1992, *Prot. Eng. 5*, 583-591) directly to the tumour.

Example 4 : Segmented human apomyoglobin as an assembly device with three segments

To use more than two segments of a native structure as an assembly device, the hydrophobic interface between the segments has to be large enough to provide the sufficient hydrophobic interaction for non-covalent linkage. Myoglobin (Fig.15) is expressible in large amounts in *E. coli* (Guillemette et al., 1991, *Protein Eng. 4*, 585-592). Up to six functional domains can be assembled by a threefold segmented structure (Fig. 16, 17, 18), three at the N-termini and three at the C-termini of the segments. The presence of heme additionally stabilizes the native-like apomyoglobin fold and can be used as a switch to influence the association constant of the multi-functional complex.

Example 5: Bioactive peptides as functional domains

Certain peptides derived from amphipathic loop structures of LPS-binding proteins (Hoess et al., 1993, *EMBO J. 12*, 3351-3356) are able to neutralize endotoxin. This effect is enhanced by multivalent display of these short peptides (10-15 residues; Hoess, unpublished results). The present invention provides a method to express and assemble several of short peptides (Fig.19), fused to an assembly segment, in a multivalent complex or in combination with other functional domains. The peptides can be fused either to the N-or to the C-terminus (Fig. 20, 21) of the assembly domain via the peptide linkers.

12

Example 6: A purification tail for IMAC as a functional domain

Peptide tails consisting of histidines are able to coordinate metal ions. They are used for purification of native proteins in immobilized metal affinity chromatography (IMAC). Multivalent display of the purification tail considerably improves the maximum purity achievable by IMAC (Lindner et al., 1992, *Methods: a companion to methods in enzymology 4*, 41-56). One or more gene cassettes (Fig. 22) encoding a polyhistidine tail can be fused to the assembly segment to provide a simple and efficient purification method for multi-functional complexes.

Example 7: The platelet aggregation inhibitor decorsin as a functional domain

Decorsin, a 39 residue protein of the leech *Macrobdella decora* (Fig. 23), acts as a potent antagonist of the platelet glycoprotein IIb-IIIa (Seymour et al., 1990, *J. Biol. Chem. 265*, 10143-10147). The gene cassette encoding the decorsin can be fused C- or N-terminally to an association segment (Fig. 24, 25). In arterial thrombotic deseases, a multivalent decorsin complex combined with an anti-fibrin antibody fragment can act as a powerful antithrombotic agent.

Claims

1.   A multifunctional polypeptide comprising:

(a)   a first amino acid sequence attached to at least one functional domain;

(b)   a second amino acid sequence attached to at least one further functional domain; and

(c)   optionally, further amino acid sequences each attached to at least one further functional domain;

wherein any one or more of said amino acid sequences interacts with at least one of said amino acid sequences in a complementary fashion to form a parental, native-like tertiary or optionally quaternary structure and wherein said parental, native-like tertiary or optionally quaternary structure is derived from a single parent polypeptide.

2.   The multifunctional polypeptide according to claim 1, wherein said single parent polypeptide is ubiquitin, acyl-phosphatase, IL2, calbindin or apomyoglobin.

3.   The multifunctional polypeptide according to claim 1 or 2, wherein said parental, native-like tertiary or optionally quaternary structure is biologically active.

4.   The multifunctional polypeptide according to any one of claims 1 to 3, wherein at least one of said functional domains is a fragment derived from a member of the immunoglobulin superfamily.

5.   The multifunctional polypeptide according to claim 4, wherein two of said functional domains are fragments derived from members of the immunoglobulin superfamily.

14

6.   The multifunctional polypeptide according to claim 4 or 5, wherein said fragments are antibody fragments.

7.   The multifunctional polypeptide according to any one of claims 1 to 6, wherein at least one of said functional domains is a biologically active molecule or a derivative thereof other than a fragment derived from a member of the immunoglobulin superfamily.

8.   The multifunctional polypeptide according to any one of claims 1 to 6, wherein the folding of the amino acid sequences is stabilised by a covalent bonding.

9.   The multifunctional polypeptide according to any one of claims 1 to 8, wherein at least one of said functional domains is coupled to said amino acid sequence(s) via a flexible peptide linker.

10.   The multifunctional polypeptide according to claim 9, wherein said flexible peptide linker is an antibody hinge region.

11.   The multifunctional polypeptide according to any one of calims 1 to 10, wherein at least one of said amino acid sequences is coupled to at least one further (poly)peptide.

12.   The multifunctional polypeptide according to claim 11, wherein said further (poly)peptide is an enzyme, a toxin, a cytokine, a metal binding site, a metal binding protein, a soluble receptor, a DNA-binding domain, a transcription factor, an immunoglobulin, a bioactive peptide of 5 to 15 amino acid residues, a peptide hormone, a growth factor, a lectin, a lipoprotein, and a peptide which is able to bind to an independent binding entity.

15

13.   The multifunctional polypeptide according to any one of claims 1 to 12, wherein at least one of said amino acid sequences, functional domains or further (poly)peptide(s) is of human origin.

14.   A DNA sequence encoding an amino acid sequence and at least one functional domain and, optionally, at least one further functional (poly)peptide comprised in the multifunctional polypeptide of any one of claims 1 to 13.

15.   A vector comprising at least one DNA molecule of claim 14.

16.   The vector of claim 15, which is a bicistronic vector.

17.   A vector cassette characterised in that it comprises a DNA sequence encoding an amino acid sequence and optionally at least one further (poly)peptide comprised in the multifunctional polypeptide of any one of claims 1 to 13, and additionally at least one, preferably a singular cloning site for inserting the DNA encoding at least one further functional domain.

18.   A vector cassette characterised in that it comprises DNA sequences encoding the amino acid sequences, and optionally the further (poly)peptide(s) comprised in the multifunctional polypeptide of any one of claims 1 to 13, and suitable restriction sites for the cloning of DNA sequences encoding the functional domains, such that upon expression of the DNA sequences after the insertion of the DNA sequences encoding the functional domains into said restriction sites, in a suitable host the multifunctional polypeptide according to any one of claims 1 to 13 is formed.

19.   The vector cassette according to claim 17 or 18 characterised in that it comprises the inserted DNA sequence(s) encoding said functional domain(s).

20.   A host cell transformed with at least one vector according to claim 15 or 16, or at least one vector cassette according to claim 19.

21.   The host cell according to claim 20, which is a mammalian, perferably human, yeast, insect, plant or bacterial, preferably E. coli cell.

22.   A method for the production of a multifunctional polypeptide according to any one of claims 1 to 13, which comprises culturing the host cell according to claim 20 or 21 in a suitable medium, and recovering said multifunctional polypeptide produced by said host cell.

23.   A method for the production of a multifunctional polypeptide according to any one of claims 1 to 13 which comprises culturing at least two host cells according to claim 20 or 21 in a suitable medium, said host cells each producing only one of said first and said second amino acid sequences attached to at least one further functional domain, recovering the amino acid sequences, mixing thereof under mildly denaturing conditions and allowing in vitro folding of the multifunctional polypeptide according to any one of claims 1 to 13 from said amino acid sequences.

24.   The method according to claim 23, wherein the further amino acid sequence(s) (each) attached to at least one further functional domain are/is produced by at least one further host cell not producing said first or second amino acid sequence.

17

25.   The method according to claim 23, wherein at least one further amino acid sequence attached to at least one further functional domain is produced by the host cell according to claim 20 or 21 producing said first or second amino acid sequence.

26.   A pharmaceutical composition comprising the multifunctional polypeptide according to any one of claims 1 to 13 optionally in combination with a pharmaceutically acceptable carrier.

27.   A diagnostic composition comprising the multifunctional polypeptide according to any one of claims 1 to 13.

28.   A kit comprising at least one vector cassette according to claim 17 or 18.

Fig.1: segmented tertiary structure for a targeted hetero-association

Fig. 2 : Modular cistron encoding functional domains
         N- and/or C-terminally fused to the assembly device

Fig. 3 : protein sequence of human ubiquitin (segmented after Gly35)

```
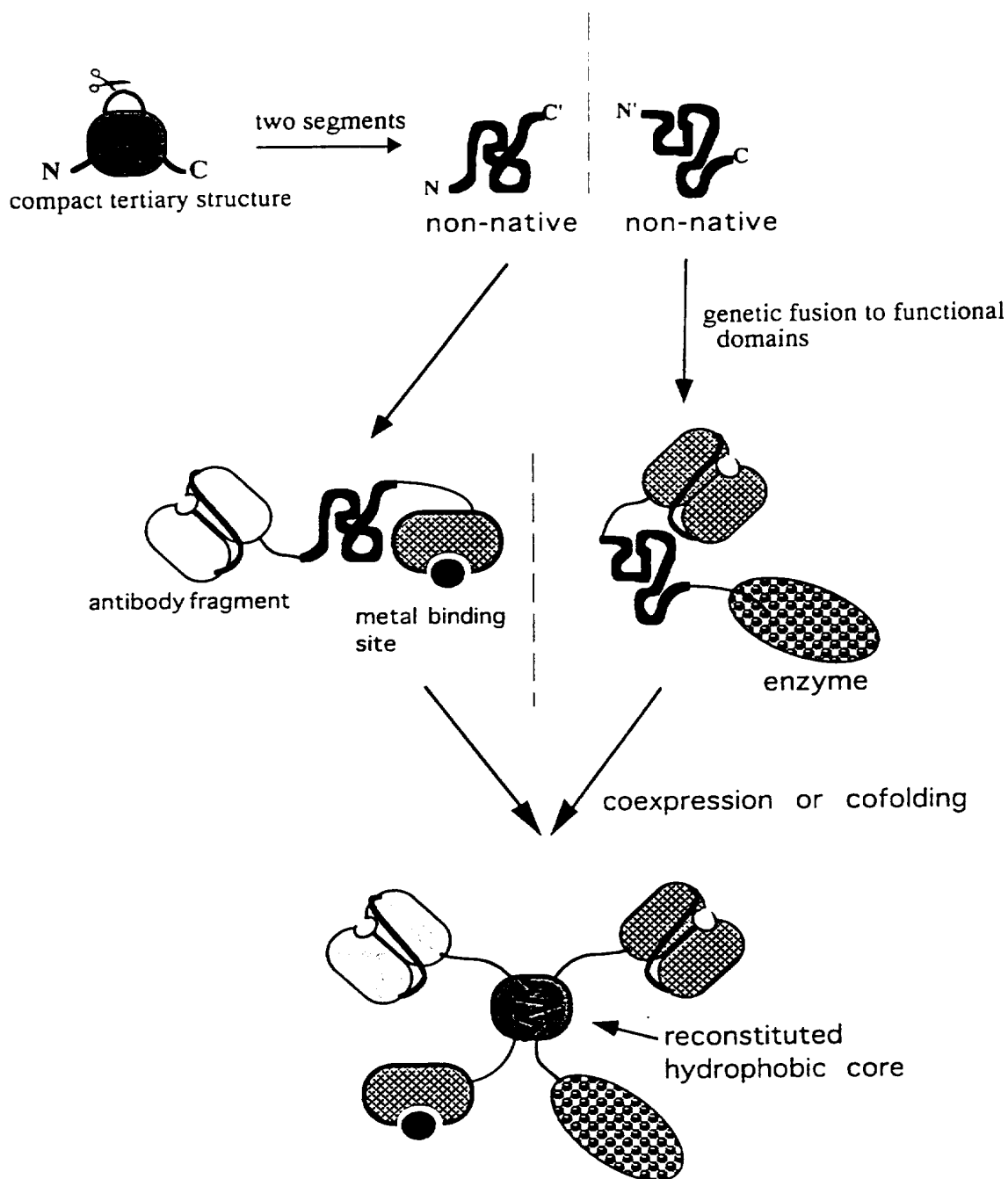1         10          20          30      ✂ 40          50
MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL
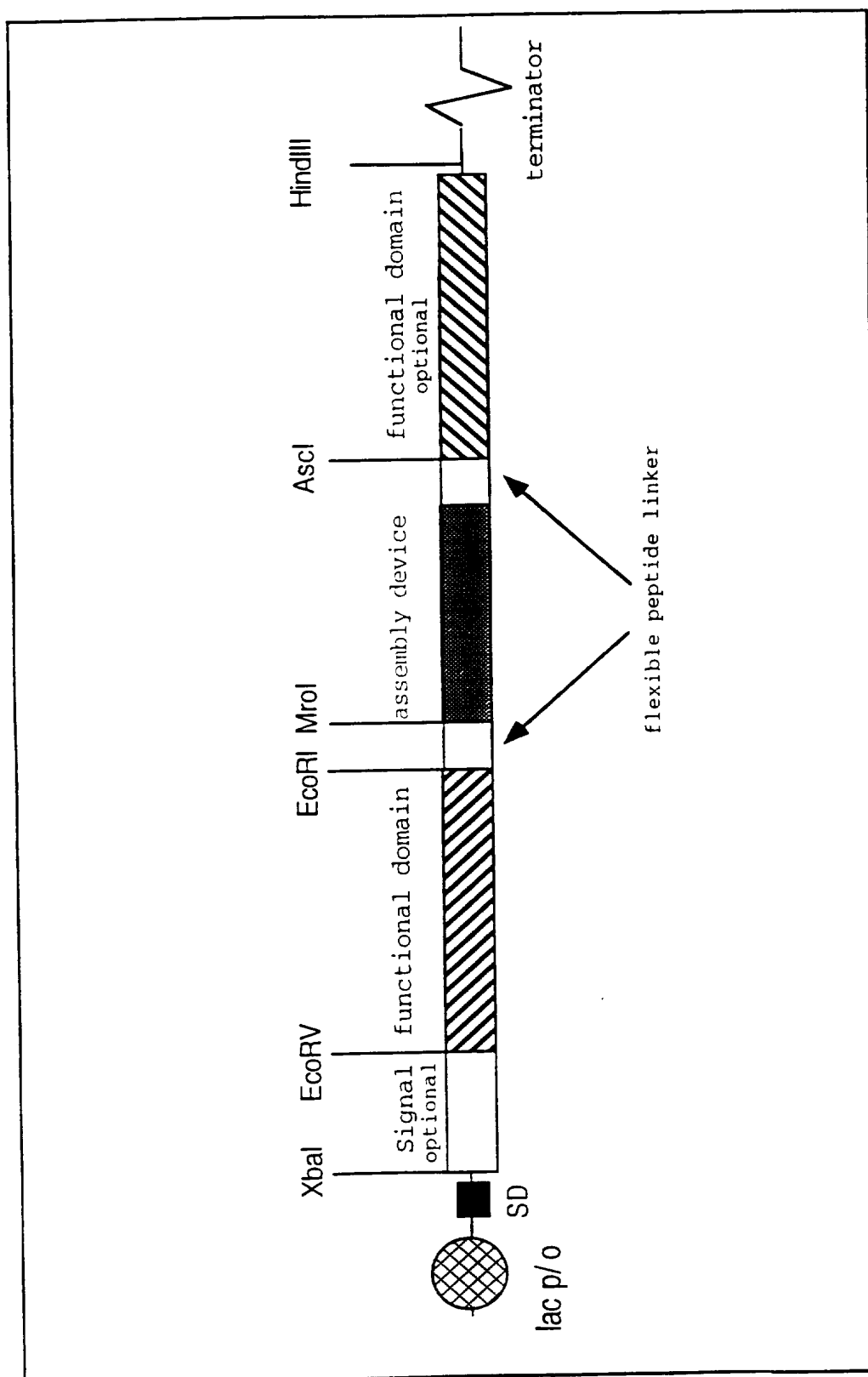

          60          70
EDGRTLSDYN IQKESTLHLV LRLRGG**
```

Fig. 4: MroI-Hind III gene cassette encoding for segment ALPHA of ubiquitin

```
MroI
S   G   M   Q   I   F   V   K   T   L   T   G   K   T   I   T   L   E
TCC GGA ATG CAG ATC TTC GTT AAA ACC CTG ACC GGT AAA ACC ATC ACC CTG GAA
        9           18          27          36          45          54
AGG CCT TAC GTC TAG AAG CAA TTT TGG GAC TGG CCA TTT TGG TAG TGG GAC CTT


V   E   P   S   D   T   I   E   N   V   K   A   K   I   Q   D   K   E
GTT GAA CCG TCT GAC ACC ATC GAA AAC GTT AAA GCT AAA ATC CAG GAC AAA GAA
        63          72          81          90          99          108
CAA CTT GGC AGA CTG TGG TAG CTT TTG CAA TTT CGA TTT TAG GTC CTG TTT CTT


          HindIII
G   *   *   A
GGT TGA TAA GCT T 3'
        117
CCA ACT ATT CGA A 5'
```

Fig. 5: MroI-Hind III gene cassette encoding for segment
BETA of ubiquitin

MroI

```
S    G    I    P    P    D    Q    Q    R    L    I    F    A    G    R    Q    L    E
TCC  GGA  ATC  CCG  CCG  GAC  CAG  CAG  CGT  CTG  ATC  TTC  GCT  GGT  CGT  CAG  CTG  GAA
          9              18             27             36             45             54
AGG  CCT  TAG  GGC  GGC  CTG  GTC  GTC  GCA  GAC  TAG  AAG  CGA  CCA  GCA  GTC  GAC  CTT
```


```
D    G    R    T    L    S    D    Y    N    I    Q    K    E    S    T    L    H    L
GAC  GGT  CGT  ACC  CTG  TCT  GAC  TAC  AAC  ATC  CAG  AAA  GAA  TCT  ACC  CTG  CAC  CTG
          63             72             81             90             99             108
CTG  CCA  GCA  TGG  GAC  AGA  CTG  ATG  TTG  TAG  GTC  TTT  CTT  AGA  TGG  GAC  GTG  GAC
```


```
                    HindIII
V    L    R    L    *    *
GTT  CTG  CGT  CTG  TGA  TAA    3'
          117            126
CAA  GAC  GCA  GAC  ACT  ATT    5'
```


Fig. 6: EcoRI-MroI gene cassette encoding a flexible linker
(huIgG3)


```
     EcoRI                                            MroI
     E    F    T    P    L    G    D    T    T    H    T    S    G
5'   GAA  TTC  ACC  CCG  CTG  GGT  GAC  ACC  ACC  CAC  ACC  TCC  GGA   3'
          9              18             27             36
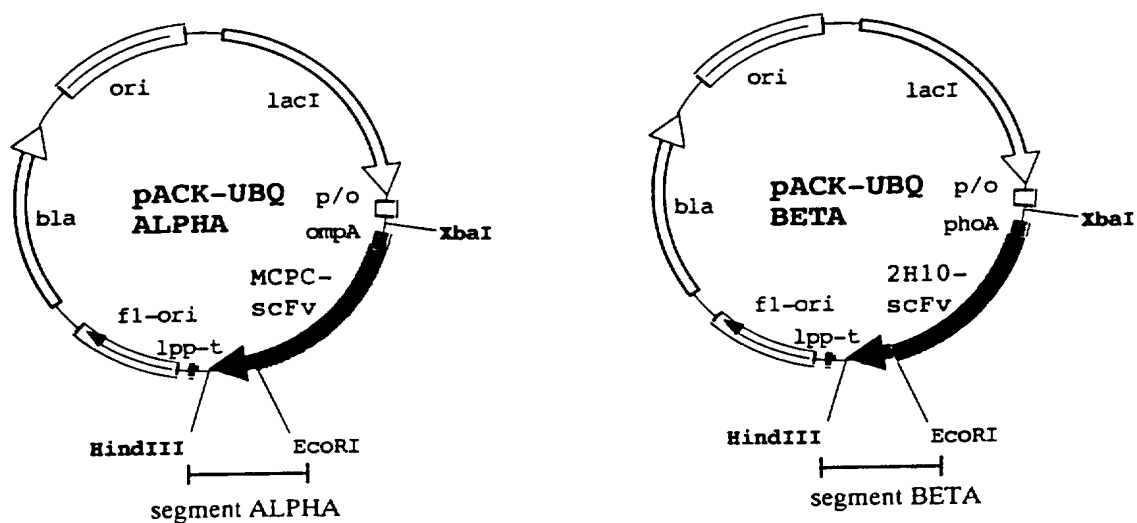3'   CTT  AAG  TGG  GGC  GAC  CCA  CTG  TGG  TGG  GTG  TGG  AGG  CCT   5'
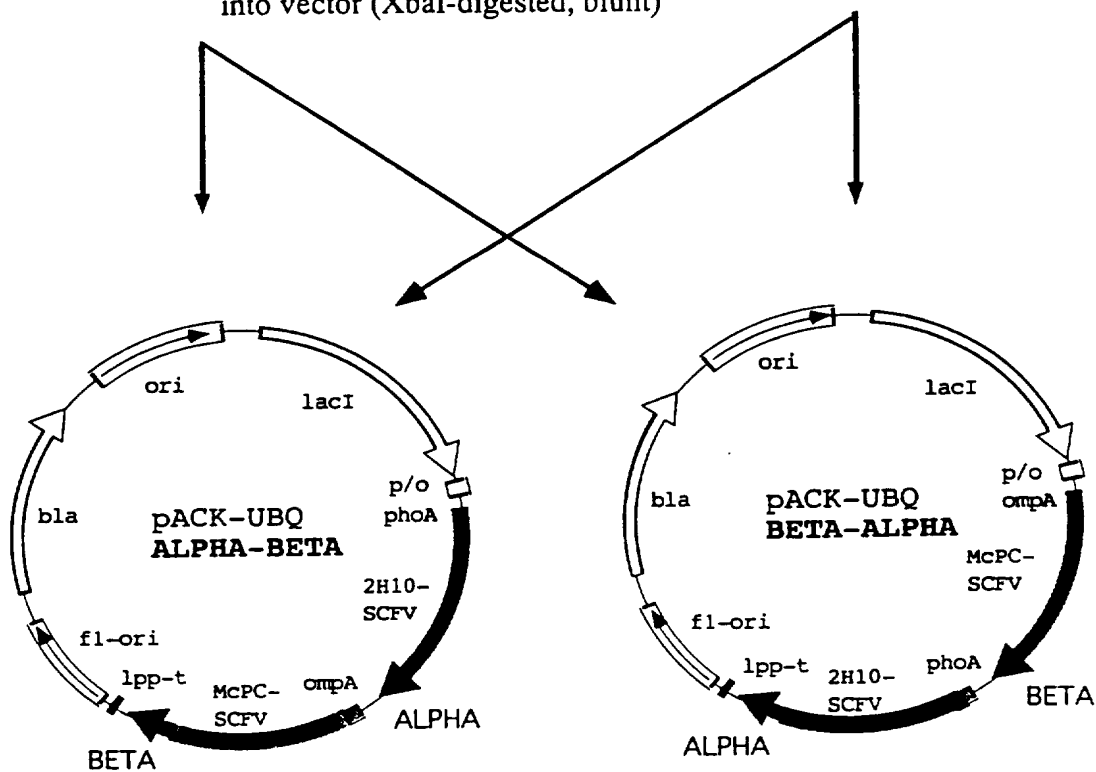```

Fig. 7 : Construction of monocistronic expression vector

6/15

Fig. 8: Construction of dicistronic co-expression vectors



insert second cistron (XbaI/HindIII-digested,blunt)
into vector (XbaI-digested, blunt)

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Fig. 9 : protein sequence of human ubiquitin with intersegmental disufides Cys4 and Cys66 (segmented after Gly35)

```
1         10        20        30      ⊱  40        50
MQICVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL
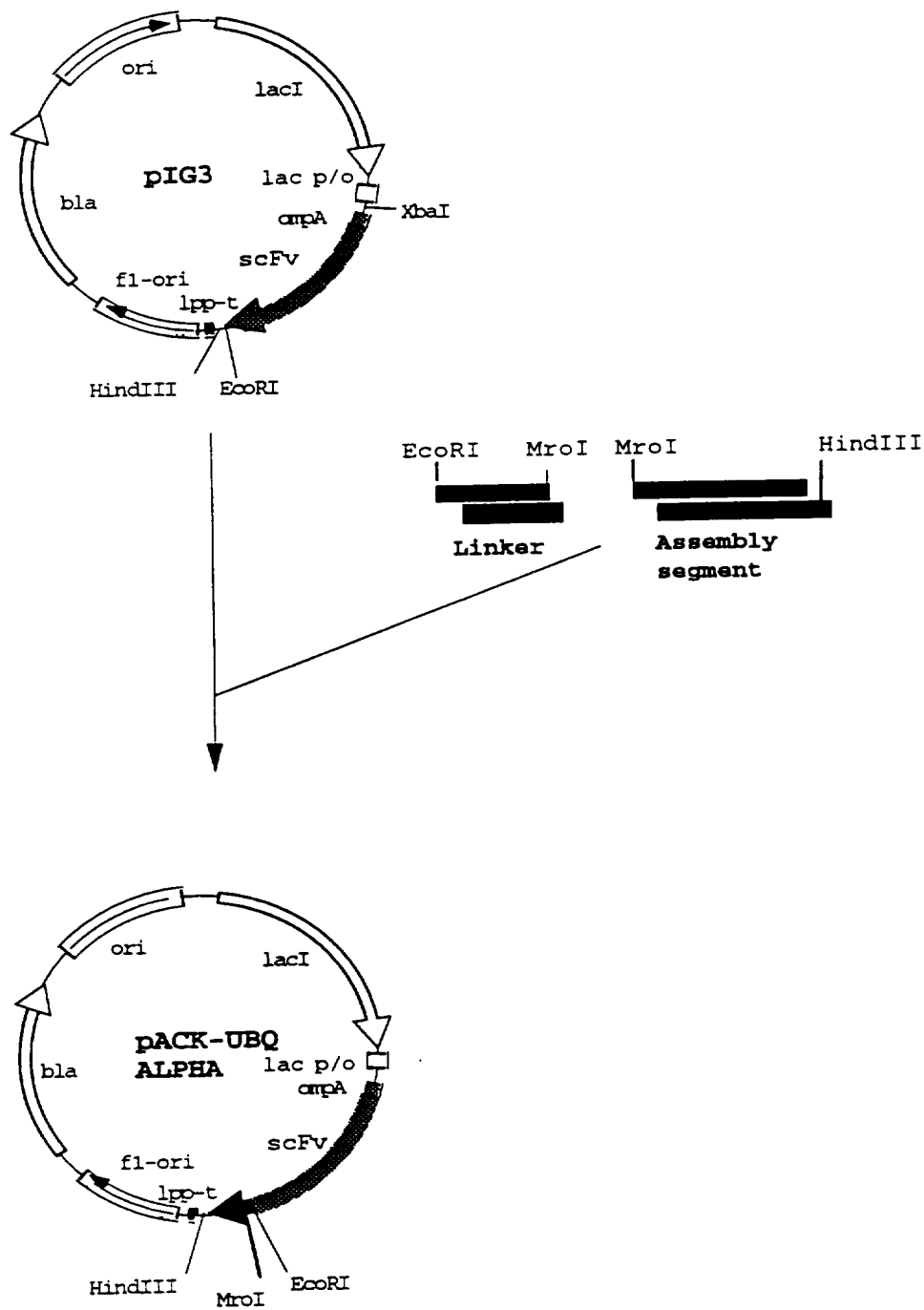

          60        70
EDGRTLSDYN IQKESCLHLV LRLRGG**
```

Fig. 10: MroI-Hind III gene cassette encoding for segment ALPHA-CYS4 of ubiquitin

MroI

```
  S   G   M   Q   I   C   V   K   T   L   T   G   K   T   I   T   L   E
 TCC GGA ATG CAG ATC TGC GTT AAA ACC CTG ACC GGT AAA ACC ATC ACC CTG GAA
      9          18          27          36          45          54
 AGG CCT TAC GTC TAG ACG CAA TTT TGG GAC TGG CCA TTT TGG TAG TGG GAC CTT


  V   E   P   S   D   T   I   E   N   V   K   A   K   I   Q   D   K   E
 GTT GAA CCG TCT GAC ACC ATC GAA AAC GTT AAA GCT AAA ATC CAG GAC AAA GAA
              63          72          81          90          99         108
 CAA CTT GGC AGA CTG TGG TAG CTT TTG CAA TTT CGA TTT TAG GTC CTG TTT CTT
```

HindIII
```
  G   *   *   A
 GGT TGA TAA GCT T 3'
          117
 CCA ACT ATT CGA A 5'
```

Fig. 11 : MroI-AscI-Hind III gene cassette encoding for segment BETA-CYS66 with C-terminal GGSGGAP linker of ubiquitin

MroI
```
S   G   I   P   P   D   Q   Q   R   L   I   F   A   G   R   Q   L   E
TCC GGA ATC CCG CCG GAC CAG CAG CGT CTG ATC TTC GCT GGT CGT CAG CTG GAA
         9           18          27          36          45          54
AGG CCT TAG GGC GGC CTG GTC GTC GCA GAC TAG AAG CGA CCA GCA GTC GAC CTT
```

```
D   G   R   T   L   S   D   Y   N   I   Q   K   E   S   C   L   H   L
GAC GGT CGT ACC CTG TCT GAC TAC AAC ATC CAG AAA GAA TCT TGC CTG CAC CTG
         63          72          81          90          99          108
CTG CCA GCA TGG GAC AGA CTG ATG TTG TAG GTC TTT CTT AGA ACG GAC GTG GAC
```

```
                                            AscI            HindIII
                                             *               *
V   L   R   L   G   G   S   G   G   A   P   *   *
GTT CTG CGT CTG GGG GGG AGC GGA GGC GCG CCG TGA TAA    3'
         117         126
CAA GAC GCA GAC CCC CCC TCG CCT CCG CGC GGC ACT ATT    5'
```

Fig. 12: Protein sequence of human IL-2 (segmented after His79)

```
        10          20          30          40          50          60
APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML TFKFYMPKKA TELKHLQCLE

        70          ✁          90         100         110         120
EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR

        130
WITFCQSIIS TLT
```

Fig.13 : MroI-AscI-Hind III gene cassette encoding for segment ALPHA of human IL-2

MroI

S	G	A	P	T	S	S	S	T	K	K	T	Q	L	Q	L	E	H
TCC	GGA	GCA	CCT	ACT	TCA	AGT	TCT	ACA	AAG	AAA	ACA	CAG	CTA	CAA	CTG	GAG	CAT

          9   18   27   36   45   54

AGG CCT CGT GGA TGA AGT TCA AGA TGT TTC TTT TGT GTC GAT GTT GAC CTC GTA

L	L	L	D	L	Q	M	I	L	N	G	I	N	N	Y	K	N	P
TTA	CTG	CTG	GAT	TTA	CAG	ATG	ATT	TTG	AAT	GGA	ATT	AAT	AAT	TAC	AAG	AAT	CCC

        63   72   81   90   99   108

AAT GAC GAC CTA AAT GTC TAC TAA AAC TTA CCT TAA TTA TTA ATG TTC TTA GGG

K	L	T	R	M	L	T	F	K	F	Y	M	P	K	K	A	T	E
AAA	CTC	ACC	AGG	ATG	CTC	ACA	TTT	AAG	TTT	TAC	ATG	CCC	AAG	AAG	GCC	ACA	GAA

        117   126   135   144   153   162

TTT GAG TGG TCC TAC GAG TGT AAA TTC AAA ATG TAC GGG TTC TTC CGG TGT CTT

L	K	H	L	Q	C	L	E	E	E	L	K	P	L	E	E	V	L
CTG	AAA	CAT	CTT	CAG	TGT	CTA	GAA	GAA	GAA	CTC	AAA	CCT	CTG	GAG	GAA	GTG	CTA

        171   180   189   198   207   216

GAC TTT GTA GAA GTC ACA GAT CTT CTT CTT GAG TTT GGA GAC CTC CTT CAC GAT

                                        AscI   **HindIII**

N	L	A	Q	S	K	N	F	H	G	G	S	G	G	A	P	*	
AAT	TTA	GCT	CAA	AGC	AAA	AAC	TTT	CAC	GGG	GGG	AGC	GGA	GGC	GCG	CCG	TGA	T

        225   234   243   252   261

TTA AAT CGA GTT TCG TTT TTG AAA GTG CCC CCC TCG CCT CCG CGC GGC ACT A

Fig. 14 : MroI-AscI-Hind III gene cassette encoding for
segment BETA of human IL-2

```
MroI
S    G    L    R    P    R    D    L    I    S    N    I    N    V    I    V    L    E
TCC  GGA  TTA  AGA  CCC  AGG  GAC  TTA  ATC  AGC  AAT  ATC  AAC  GTA  ATA  GTT  CTG  GAA
     9              18             27             36             45                  54
AGG  CCT  AAT  TCT  GGG  TCC  CTG  AAT  TAG  TCG  TTA  TAG  TTG  CAT  TAT  CAA  GAC  CTT


L    K    G    S    E    T    T    F    M    C    E    Y    A    D    E    T    A    T
CTA  AAG  GGA  TCT  GAA  ACA  ACA  TTC  ATG  TGT  GAA  TAT  GCT  GAT  GAG  ACA  GCA  ACC
          63             72             81             90             99             108
GAT  TTC  CCT  AGA  CTT  TGT  TGT  AAG  TAC  ACA  CTT  ATA  CGA  CTA  CTC  TGT  CGT  TGG


I    V    E    F    L    N    R    W    I    T    F    C    Q    S    I    I    S    T
ATT  GTA  GAA  TTT  CTG  AAC  AGA  TGG  ATT  ACC  TTT  TGT  CAA  AGC  ATC  ATC  TCA  ACA
          117            126            135            144            153            162
TAA  CAT  CTT  AAA  GAC  TTG  TCT  ACC  TAA  TGG  AAA  ACA  GTT  TCG  TAG  TAG  AGT  TGT


                         AscI           HindIII
L    T    G    G    S    G    G    A    P    *
CTG  ACT  GGG  GGG  AGC  GGA  GGC  GCG  CCG  TGA  T 3'
          171            180            189
GAC  TGA  CCC  CCC  TCG  CCT  CCG  CGC  GGC  ACT  A 5'
```

Fig. 15 Protein sequence of human apomyoglobin (cut after Lys47 and Lys98)

    mglsdgewql vlnvwgkvea dipghgqevl irlfkghpet lekfdkfkhl

    51
    ksedemkase dlkkhgatvl talggilkkk ghheaeikpl aqshatkhki

    101
    pvkylefise ciiqvlqskh pgdfgadaeg amnkalelfr kdmasnykel

    151
    gfqg

Fig. 16 : MroI-AscI-Hind III gene cassette encoding for segment ALPHA of human apomyoglobin

MroI
S   G   M   G   L   S   D   G   E   W   Q   L   V   L   N   V   W   G
TCC GGA ATG GGT CTG TCT GAC GGT GAA TGG CAG CTG GTT CTG AAC GTT TGG GGT
        9   18   27   36   45   54
AGG CCT TAC CCA GAC AGA CTG CCA CTT ACC GTC GAC CAA GAC TTG CAA ACC CCA

K   V   E   A   D   I   P   G   H   G   Q   E   V   L   I   R   L   F
AAA GTT GAA GCT GAC ATC CCG GGT CAC GGT CAG GAA GTT CTG ATC CGT CTG TTC
        63   72   81   90   99   108
TTT CAA CTT CGA CTG TAG GGC CCA GTG CCA GTC CTT CAA GAC TAG GCA GAC AAG

K   G   H   P   E   T   L   E   K   F   D   K   F   K   G   G   S   G
AAA GGT CAC CCG GAA ACC CTG GAA AAA TTC GAC AAA TTC AAA GGG GGG AGC GGA
        117   126   135   144   153   162
TTT CCA GTG GGC CTT TGG GAC CTT TTT AAG CTG TTT AAG TTT CCC CCC TCG CCT

AscI   HindIII
G   A   P   *
GGC GCG CCG TGA T 3'
        171
CCG CGC GGC ACT A 5'

Fig. 17: MroI-AscI-Hind III gene cassette encoding for segment BETA of human apomyoglobin

```
MroI
  S    G    H    L    K    S    E    D    E    M    K    A    S    E    D    L    K    K
 TCC  GGA  CAC  CTG  AAA  TCT  GAA  GAC  GAA  ATG  AAA  GCA  TCT  GAA  GAC  CTG  AAA  AAA
           9              18             27             36             45             54
 AGG  CCT  GTG  GAC  TTT  AGA  CTT  CTG  CTT  TAC  TTT  CGT  AGA  CTT  CTG  GAC  TTT  TTT


  H    G    A    T    V    L    T    A    L    G    G    I    L    K    K    K    G    H
 CAC  GGT  GCT  ACC  GTT  CTG  ACC  GCT  CTG  GGT  GGT  ATC  CTG  AAA  AAA  AAA  GGT  CAC
           63             72             81             90             99            108
 GTG  CCA  CGA  TGG  CAA  GAC  TGG  CGA  GAC  CCA  CCA  TAG  GAC  TTT  TTT  TTT  CCA  GTG


  H    E    A    E    I    K    P    L    A    Q    S    H    A    T    K    H    K    G
 CAC  GAA  GCT  GAA  ATC  AAA  CCG  CTG  GCT  CAG  TCT  CAC  GCT  ACC  AAA  CAC  AAA  GGG
           117           126            135            144            153           162
 GTG  CTT  CGA  CTT  TAG  TTT  GGC  GAC  CGA  GTC  AGA  GTG  CGA  TGG  TTT  GTG  TTT  CCC


                 AscI              HindIII
  G    S    G    G    A    P    *
 GGG  AGC  GGA  GGC  GCG  CCG  TGA  T 3'
           171            180
 CCC  TCG  CCT  CCG  CGC  GGC  ACT  A 5'
```

Fig.18: MroI-AscI-Hind III gene cassette encoding for segment GAMMA of human apomyoglobin

```
MroI
 S    G    I    P    V    K    Y    L    E    F    I    S    E    C    I    I    Q    V
TCC  GGA  ATC  CCG  GTT  AAA  TAC  CTG  GAG  TTC  ATC  TCT  GAA  TGC  ATC  ATC  CAG  GTT
          9              18             27             36             45             54
AGG  CCT  TAG  GGC  CAA  TTT  ATG  GAC  CTC  AAG  TAG  AGA  CTT  ACG  TAG  TAG  GTC  CAA


 L    Q    S    K    H    P    G    D    F    G    A    D    A    E    G    A    M    N
CTG  CAG  TCT  AAA  CAC  CCG  GGT  GAC  TTC  GGT  GCT  GAC  GCT  GAA  GGT  GCT  ATG  AAC
          63             72             81             90             99             108
GAC  GTC  AGA  TTT  GTG  GGC  CCA  CTG  AAG  CCA  CGA  CTG  CGA  CTT  CCA  CGA  TAC  TTG


 K    A    L    E    L    F    R    K    D    M    A    S    N    Y    K    E    L    G
AAA  GCT  CTG  GAA  CTG  TTC  CGT  AAA  GAC  ATG  GCT  TCT  AAC  TAC  AAA  GAA  CTG  GGT
          117            126            135            144            153            162
TTT  CGA  GAC  CTT  GAC  AAG  GCA  TTT  CTG  TAC  CGA  AGA  TTG  ATG  TTT  CTT  GAC  CCA
 F    S    Q    F    Q    E    T    F    V    H    S    R    V    V    F    F    Q    T
                                        AscI            HindIII
 F    Q    G    G    G    S    G    G    A    P    *
TTC  CAG  GGT  GGG  GGG  AGC  GGA  GGC  GCG  CCG  TGA  T 3'
          171            180            189
AAG  GTC  CCA  CCC  CCC  TCG  CCT  CCG  CGC  GGC  ACT  A 5'
```

14/15

Fig. 19: Peptide sequence of an endotoxin-neutralizing peptide as a functional domain

```
1                11
RWKVRKSFFKL Q
```

Fig.20: N-terminal EcoRV-EcoRI cassette encoding an endotoxin-neutralizing peptide

```
   EcoRV                                                        EcoRI
    I    M    R    W    K    V    R    K    S    F    F    K    L    Q    E    F
5' ATC  ATG  CGT  TGG  AAA  GTT  CGT  AAA  TCT  TTC  TTC  AAA  CTG  CAG  GAA  TTC  3'
              9              18             27             36             45
3' TAG  TAC  GCA  ACC  TTT  CAA  GCA  TTT  AGA  AAG  AAG  TTT  GAC  GTC  CTT  AAG  5'
```

Fig.21: C-terminal AscI-HindIII cassette encoding an endotoxin-neutralizing peptide

```
   AscI                                                        HindIII
    A    P    R    W    K    V    R    K    S    F    F    K    L    Q    *    *
5' GCG  CCG  CGT  TGG  AAA  GTT  CGT  AAA  TCT  TTC  TTC  AAA  CTG  CAG  TGA  TAA  3'
              9              18             27             36             45
3' CGC  GGC  GCA  ACC  TTT  CAA  GCA  TTT  AGA  AAG  AAG  TTT  GAC  GTC  ACT  ATT  5'
```

Fig. 22 AscI-HINDIII Gene cassette encoding a purification tail for IMAC

```
   AscI               Hind III
    A    P    H    H    H    H    H    H    *    *
5' GCG  CCG  CAC  CAC  CAC  CAC  CAC  CAC  TGA  TAA  3'
              9              18             27
3' CGC  GGC  GTG  GTG  GTG  GTG  GTG  CAC  ACT  ATT  5'
```

Fig. 23   Protein sequence of the platelet aggregation
inhibitor decorsin as a functional domain


```
    1          11         21         31
    APRLPQCQGD DQEKCLCNKD ECPPGQCRFP RGDADPYCE
```


Fig. 24   N-terminal EcoRV-EcoRI cassette encoding the
platelet aggregation inhibitor decorsin


```
EcoRV
D   I   A   P   R   L   P   Q   C   Q   G   D   D   Q   E   K   C   L
GAT ATC GCT CCG CGT CTG CCG CAG TGC CAG GGT GAC GAC CAG GAA AAA TGC CTG
        9           18          27          36          45          54
CTA TAG CGA GGC GCA GAC GGC GTC ACG GTC CCA CTG CTG GTC CTT TTT ACG GAC


C   N   K   D   E   C   P   P   G   Q   C   R   F   P   R   G   D   A
TGC AAC AAA GAC GAA TGC CCG CCG GGT CAG TGC CGT TTC CCG CGT GGT GAC GCT
        63          72          81          90          99          108
ACG TTG TTT CTG CTT ACG GGC GGC CCA GTC ACG GCA AAG GGC GCA CCA CTG CGA


                EcoRI
D   P   Y   C   E   F
GAC CCG TAC TGC GAA TTC   3'
        117         126
CTG GGC ATG ACG CTT AAG   5'
```


Fig. 25   C-terminal AscI-HindIII cassette encoding the
platelet aggregation inhibitor decorsin


```
AscI
A   P   A   P   R   L   P   Q   C   Q   G   D   D   Q   E   K   C   L
GCG CCG GCT CCG CGT CTG CCG CAG TGC CAG GGT GAC GAC CAG GAA AAA TGC CTG
        12          21          30          39          48          57
CGC GGC CGA GGC GCA GAC GGC GTC ACG GTC CCA CTG CTG GTC CTT TTT ACG GAC


C   N   K   D   E   C   P   P   G   Q   C   R   F   P   R   G   D   A
TGC AAC AAA GAC GAA TGC CCG CCG GGT CAG TGC CGT TTC CCG CGT GGT GAC GCT
        66          75          84          93          102         111
ACG TTG TTT CTG CTT ACG GGC GGC CCA GTC ACG GCA AAG GGC GCA CCA CTG CGA


                HindIII
D   P   Y   C   E   *   *
GAC CCG TAC TGC GAA TGA TAA   3'
        120         129
CTG GGC ATG ACG CTT ACT ATT   5'
```

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(54) Title: MODULATORS OF AMYLOID AGGREGATION

(57) Abstract

Compounds that modulate the aggregation of amyloidogenic proteins or peptides are disclosed. The modulators of the invention can promote amyloid aggregation or, more preferably, can inhibit natural amyloid aggregation. In a preferred embodiment, the compounds modulate the aggregation of natural β amyloid peptides (β-AP). In a preferred embodiment, the β amyloid modulator compounds of the invention are comprised of an Aβ aggregation core domain and a modifying group coupled thereto such that the compound alters the aggregation or inhibits the neurotoxicity of natural β amyloid peptides when contacted with the peptides. Furthermore, the modulators are capable of altering natural β-AP aggregation when the natural β-APs are in a molar excess amount relative to the modulators. Pharmaceutical compositions comprising the compounds of the invention, and diagnostic and treatment methods for amyloidogenic diseases using the compounds of the invention, are also disclosed.

MODULATORS OF AMYLOID AGGREGATION

Background of the Invention

Alzheimer's disease (AD), first described by the Bavarian psychiatrist Alois
5   Alzheimer in 1907, is a progressive neurological disorder that begins with short term memory
loss and proceeds to disorientation, impairment of judgement and reasoning and, ultimately,
dementia. The course of the disease usually leads to death in a severely debilitated, immobile
state between four and 12 years after onset. AD has been estimated to afflict 5 to 11 percent
of the population over age 65 and as much as 47 percent of the population over age 85. The
10   societal cost for managing AD is upwards of 80 billion dollars annually, primarily due to the
extensive custodial care required for AD patients. Moreover, as adults born during the
population boom of the 1940's and 1950's approach the age when AD becomes more
prevalent, the control and treatment of AD will become an even more significant health care
problem. Currently, there is no treatment that significantly retards the progression of the
15   disease. For reviews on AD, see Selkoe, D.J. *Sci. Amer.* , November 1991, pp. 68-78; and
Yankner, B.A. *et al.* (1991) *N. Eng. J. Med.* 325:1849-1857.

It has recently been reported (Games *et al.* (1995) *Nature* 373:523-527) that an
Alzheimer-type neuropathology has been created in transgenic mice. The transgenic mice
express high levels of human mutant amyloid precursor protein and progressively develop
20   many of the pathological conditions associated with AD.

Pathologically, AD is characterized by the presence of distinctive lesions in the
victim's brain. These brain lesions include abnormal intracellular filaments called
neurofibrillary tangles (NTFs) and extracellular deposits of amyloidogenic proteins in senile,
or amyloid, plaques. Amyloid deposits are also present in the walls of cerebral blood vessels
25   of AD patients. The major protein constituent of amyloid plaques has been identified as a 4
kilodalton peptide called β-amyloid peptide (β-AP)(Glenner, G.G. and Wong, C.W. (1984)
Biochem. Biophys. Res. Commun. 120:885-890; Masters, C. *et al.* (1985) *Proc. Natl. Acad.
Sci. USA* 82:4245-4249). Diffuse deposits of β-AP are frequently observed in normal adult
brains, whereas AD brain tissue is characterized by more compacted, dense-core β-amyloid
30   plaques. (See *e.g.,* Davies, L. *et al.* (1988) *Neurology* 38:1688-1693) These observations
suggest that β-AP deposition precedes, and contributes to, the destruction of neurons that
occurs in AD. In further support of a direct pathogenic role for β-AP, β-amyloid has been
shown to be toxic to mature neurons, both in culture and *in vivo.* Yankner, B.A. *et al.* (1989)
Science 245:417-420; Yankner, B.A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:9020-9023;
35   Roher, A.E. *et al.* (1991) *Biochem. Biophys. Res. Commun.* 174:572-579; Kowall, N.W. *et al.*
(1991) *Proc. Natl. Acad. Sci. USA* 88:7247-7251. Furthermore, patients with hereditary
cerebral hemorrhage with amyloidosis-Dutch-type (HCHWA-D), which is characterized by
diffuse β-amyloid deposits within the cerebral cortex and cerebrovasculature, have been
shown to have a point mutation that leads to an amino acid substitution within β-AP. Levy,

E. *et al.* (1990) *Science* 248:1124-1126. This²observation demonstrates that a specific alteration of the β-AP sequence can cause β-amyloid to be deposited.

Natural β-AP is derived by proteolysis from a much larger protein called the amyloid precursor protein (APP). Kang, J. *et al.* (1987) *Nature* 325:733; Goldgaber, D. *et al.* (1987)

5   *Science* 235:877; Robakis, N.K. *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4190; Tanzi, R.E. *et al.* (1987) *Science* 235:880. The APP gene maps to chromosome 21, thereby providing an explanation for the β-amyloid deposition seen at an early age in individuals with Down's syndrome, which is caused by trisomy of chromosome 21. Mann, D.M. *et al.* (1989) *Neuropathol. Appl. Neurobiol.* 15:317; Rumble, B. *et al.* (1989) *N. Eng. J. Med.* 320:1446.

10   APP contains a single membrane spanning domain, with a long amino terminal region (about two-thirds of the protein) extending into the extracellular environment and a shorter carboxy-terminal region projecting into the cytoplasm. Differential splicing of the APP messenger RNA leads to at least five forms of APP, composed of either 563 amino acids (APP-563), 695 amino acids (APP-695), 714 amino acids (APP-714), 751 amino acids (APP-751) or 770

15   amino acids (APP-770).

Within APP, naturally-occurring β amyloid peptide begins at an aspartic acid residue at amino acid position 672 of APP-770. Naturally-occurring β-AP derived from proteolysis of APP is 39 to 43 amino acid residues in length, depending on the carboxy-terminal end point, which exhibits heterogeneity. The predominant circulating form of β-AP in the blood

20   and cerebrospinal fluid of both AD patients and normal adults is β1-40 ("short β"). Seubert, P. *et al.* (1992) *Nature* 359:325; Shoji, M. *et al.* (1992) *Science* 258:126. However, β1-42 and β1-43 ("long β") also are forms in β-amyloid plaques. Masters, C. *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:4245; Miller, D. *et al.* (1993) *Arch. Biochem. Biophys.* 301:41; Mori, H. *et al.* (1992) *J. Biol. Chem.* 267:17082. Although the precise molecular mechanism

25   leading to β-APP aggregation and deposition is unknown, the process has been likened to that of nucleation-dependent polymerizations, such as protein crystallization, microtubule formation and actin polymerization. See *e.g.*, Jarrett, J.T. and Lansbury, P.T. (1993) *Cell* 73:1055-1058. In such processes, polymerization of monomer components does not occur until nucleus formation. Thus, these processes are characterized by a lag time before

30   aggregation occurs, followed by rapid polymerization after nucleation. Nucleation can be accelerated by the addition of a "seed" or preformed nucleus, which results in rapid polymerization. The long β forms of β-AP have been shown to act as seeds, thereby accelerating polymerization of both long and short β-AP forms. Jarrett, J.T. *et al.* (1993) *Biochemistry* 32:4693.

35   In one study, in which amino acid substitutions were made in β-AP, two mutant β peptides were reported to interfere with polymerization of non-mutated β-AP when the mutant and non-mutant forms of peptide were mixed. Hilbich, C. *et al.* (1992) *J. Mol. Biol.* 228:460-473. However, equimolar amounts of the mutant and non-mutant (*i.e.*, natural) β

amyloid peptides were used to see this effect ³and the mutant peptides were reported to be unsuitable for use *in vivo*. Hilbich, C. *et al.* (1992), *supra*.

Summary of the Invention

5   This invention pertains to compounds, and pharmaceutical compositions thereof, that can modulate the aggregation of amyloidogenic proteins and peptides, in particular compounds that can modulate the aggregation of natural β amyloid peptides (β-AP) and inhibit the neurotoxicity of natural β-APs. In one embodiment, the invention provides an amyloid modulator compound comprising an amyloidogenic protein, or peptide fragment

10   thereof, coupled directly or indirectly to at least one modifying group such that the compound modulates the aggregation of natural amyloid proteins or peptides when contacted with the natural amyloidogenic proteins or peptides. Preferably, the compound inhibits aggregation of natural amyloidogenic proteins or peptides when contacted with the natural amyloidogenic proteins or peptides. The amyloidogenic protein, or peptide fragment thereof. can be. for

15   example. selected from the group consisting of transthyretin (TTR), prion protein (PrP). islet amyloid polypeptide (IAPP), atrial natriuretic factor (ANF), kappa light chain. lambda light chain, amyloid A, procalcitonin, cystatin C, β2 microglobulin, ApoA-I. gelsolin, procalcitonin, calcitonin, fibrinogen and lysozyme.

          In the most preferred embodiment of the invention, the compound modulates the

20   aggregation of natural β-AP. The invention provides a β-amyloid peptide compound comprising a formula:

$$(\text{Xaa})_n^{\text{An}}$$

25   wherein Xaa is a β-amyloid peptide having an amino-terminal amino acid residue corresponding to position 668 of β-amyloid precursor protein-770 (APP-770) or to a residue carboxy-terminal to position 668 of APP-770, A is a modifying group attached directly or indirectly to the β-amyloid peptide of the compound such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid

30   peptides. and n is an integer selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

          In one embodiment, at least one A group is attached directly or indirectly to the amino terminus of the β-amyloid peptide of the compound. In another embodiment. at least one A group is attached directly or indirectly to the carboxy terminus of the β-amyloid peptide of

35   the compound. In yet another embodiment, at least one A group is attached directly or indirectly to a side chain of at least one amino acid residue of the β-amyloid peptide of the compound.

4

The invention also provides a β-amyloid modulator compound comprising an Aβ aggregation core domain (ACD) coupled directly or indirectly to at least one modifying group (MG) such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

5   Preferably, the Aβ aggregation core domain is modeled after a subregion of natural β-amyloid peptide between 3 and 10 amino acids in length.

The invention also provides β-amyloid modulator compound comprising a formula:

$$(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Z)_n^{A}$$

10

wherein Xaa_1, Xaa_2 and Xaa_3 are each amino acid structures and at least two of Xaa_1, Xaa_2 and Xaa_3 are. independently. selected from the group consisting of a leucine structure, a phenylalanine structure and a valine structure;

Y. which may or may not be present. is a peptidic structure having the

15   formula $(Xaa)_a$, wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

Z. which may or may not be present, is a peptidic structure having the formula $(Xaa)_b$, wherein Xaa is any amino acid structure and b is an integer from 1 to 15; and

A is a modifying group attached directly or indirectly to the compound and n is an integer;

20   Xaa_1, Xaa_2. Xaa_3. Y. Z. A and n being selected such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. In a preferred embodiment. Xaa_1 and Xaa_2 are each phenylalanine structures. In another preferred embodiment Xaa_2 and Xaa_3 are each phenylalanine structures.

25   The invention further provides a β-amyloid modulator compound comprising a formula:

$$(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}Z)_n^{A}$$

30   wherein Xaa_1 and Xaa_3 are amino acid structures;

Xaa_2 is a valine structure;

Xaa_4 is a phenylalanine structure;

Y. which may or may not be present, is a peptidic structure having the formula $(Xaa)_a$, wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

35   Z. which may or may not be present. is a peptidic structure having the formula $(Xaa)_b$, wherein Xaa is any amino acid structure and b is an integer from 1 to 15: and

5

A is a modifying group attached directly or indirectly to the compound and n is an integer;

Xaa_1, Xaa_3, Y, Z, A and n being selected such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. In a preferred embodiment, Xaa_1 is a leucine structure and Xaa_3 is phenylalanine structure.

The invention still further provides a compound comprising the formula:

$$A\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}Xaa_5\text{-}Xaa_6\text{-}Xaa_7\text{-}Xaa_8\text{-}B$$

wherein Xaa1 is a histidine structure;

      Xaa2 is a glutamine structure;

      Xaa3 is a lysine structure;

      Xaa4 is a leucine structure;

      Xaa5 is a valine structure;

      Xaa6 is a phenylalanine structure;

      Xaa7 is a phenylalanine structure;

      Xaa8 is an alanine structure;

      A and B are modifying groups attached directly or indirectly to the amino terminus and carboxy terminus, respectively, of the compound;

      and wherein Xaa_1-Xaa_2-Xaa_3, Xaa_1-Xaa_2 or Xaa_1 may or may not be present;

      Xaa_8 may or may not be present; and

      at least one of A and B is present.

The invention still further provides a β-amyloid modulator compound comprising a modifying group attached directly or indirectly to a peptidic structure, wherein the peptidic structure comprises amino acid structures having an amino acid sequence selected from the group consisting of His-Gln-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 5), His-Gln-Lys-Leu-Val-Phe-Phe (SEQ ID NO: 6), Gln-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 7), Gln-Lys-Leu-Val-Phe-Phe (SEQ ID NO: 8), Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 9), Lys-Leu-Val-Phe-Phe (SEQ ID NO: 10), Leu-Val-Phe-Phe-Ala (SEQ ID NO: 11), Leu-Val-Phe-Phe (SEQ ID NO: 12), Leu-Ala-Phe-Phe-Ala (SEQ ID NO: 13), Val-Phe-Phe (SEQ ID NO: 19), Phe-Phe-Ala (SEQ ID NO: 20), Phe-Phe-Val-Leu-Ala (SEQ ID NO: 21), Leu-Val-Phe-Phe-Lys (SEQ ID NO: 22), Leu-Val-Iodotyrosine-Phe-Ala (SEQ ID NO: 23), Val-Phe-Phe-Ala (SEQ ID NO: 24), Ala-Val-Phe-Phe-Ala (SEQ ID NO: 25), Leu-Val-Phe-Iodotyrosine-Ala (SEQ ID NO: 26), Leu-Val-Phe-Phe-Ala-Glu (SEQ ID NO: 27), Phe-Phe-Val-Leu (SEQ ID NO: 28), Phe-Lys-Phe-Val-Leu (SEQ ID NO: 29), Lys-Leu-Val-Ala-Phe (SEQ ID NO: 30), Lys-Leu-Val-Phe-Phe-βAla (SEQ ID NO: 31) and Leu-Val-Phe-Phe-DAla (SEQ ID NO: 32).

In the compounds of the invention comprising a modifying group, preferably the modifying group comprises a cyclic, heterocyclic or polycyclic group. Preferred modifying

groups contains a cis-decalin group, such as a cholanoyl structure. Preferred modifying groups include a cholyl group, a biotin-containing group, a diethylene-triaminepentaacetyl group, a (-)-menthoxyacetyl group, a fluorescein-containing group or an N-acetylneuraminyl group.

The compounds of the invention can be further modified, for example to alter a pharmacokinetic property of the compound or to label the compound with a detectable substance. Preferred radioactive labels are radioactive iodine or technetium.

The invention also provides a β-amyloid modulator which inhibits aggregation of natural β-amyloid peptides when contacted with a molar excess amount of natural β-amyloid peptides.

The invention also provides a β-amyloid peptide compound comprising an amino acid sequence having at least one amino acid deletion compared to βAP_{1-39}, such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. In one embodiment, the compound has at least one internal amino acid deleted compared to βAP_{1-39}. In another embodiment, the compound has at least one N-terminal amino acid deleted compared to βAP_{1-39}. In yet another embodiment, the compound has at least one C-terminal amino acid deleted compared to βAP_{1-39}. Preferred compounds include βAP_{6-20} (SEQ ID NO: 13), βAP_{16-30} (SEQ ID NO: 14), $\beta AP_{1-20, 26-40}$ (SEQ ID NO: 15) and EEVVHHHHQQ-βAP_{16-40} (SEQ ID NO: 16).

The compounds of the invention can be formulated into pharmaceutical compositions comprising the compound and a pharmaceutically acceptable carrier. The compounds can also be used in the manufacture of a medicament for the diagnosis or treatment of an amyloidogenic disease.

Another aspect of the invention pertains to diagnostic and treatment methods using the compounds of the invention. The invention provides a method for inhibiting aggregation of natural β-amyloid peptides, comprising contacting the natural β-amyloid peptides with a compound of the invention such that aggregation of the natural β-amyloid peptides is inhibited. The invention also provides a method for inhibiting neurotoxicity of natural β-amyloid peptides, comprising contacting the natural β-amyloid peptides with a compound of the invention such that neurotoxicity of the natural β-amyloid peptides is inhibited.

In another embodiment, the invention provides a method for detecting the presence or absence of natural β-amyloid peptides in a biological sample, comprising contacting a biological sample with a compound of the invention and detecting the compound bound to natural β-amyloid peptides to thereby detect the presence or absence of natural β-amyloid peptides in the biological sample. In one embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine.

7

In another embodiment, the invention provides a method for detecting natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease, comprising contacting a biological sample with a compound of the invention and detecting the compound bound to natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease. In one

5 embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine. Preferably, the method facilitates diagnosis of Alzheimer's disease.

10   The invention also provides a method for treating a subject for a disorder associated with amyloidosis, comprising administering to the subject a therapeutically or prophylactically effective amount of a compound of the invention such that the subject is treated for a disorder associated with amyloidosis. The method can be used to treat disorders is selected, for example, from the group consisting of familial amyloid polyneuropathy

15 (Portuguese, Japanese and Swedish types), familial amyloid cardiomyopathy (Danish type), isolated cardiac amyloid, systemic senile amyloidosis, scrapie, bovine spongiform encephalopathy, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, adult onset diabetes, insulinoma, isolated atrial amyloidosis, idiopathic (primary) amyloidosis, myeloma or macroglobulinemia-associated amyloidosis, primary localized cutaneous

20 nodular amyloidosis associated with Sjogren's syndrome, reactive (secondary) amyloidosis, familial Mediterranean Fever and familial amyloid nephropathy with urticaria and deafness (Muckle-Wells syndrome), hereditary cerebral hemorrhage with amyloidosis of Icelandic type, amyloidosis associated with long term hemodialysis, hereditary non-neuropathic systemic amyloidosis (familial amyloid polyneuropathy III), familial amyloidosis of Finnish

25 type, amyloidosis associated with medullary carcinoma of the thyroid, fibrinogen-associated hereditary renal amyloidosis and lysozyme-associated hereditary systemic amyloidosis.

In a preferred embodiment, the invention provides a method for treating a subject for a disorder associated with β-amyloidosis, comprising administering to the subject a therapeutically or prophylactically effective amount of a compound of the invention such that

30 the subject is treated for a disorder associated with β-amyloidosis. Preferably the disorder is Alzheimer's disease.

In yet another embodiment, the invention provides a method for treating a subject for a disorder associated with β-amyloidosis, comprising administering to the subject a recombinant expression vector encoding a peptide compound of the invention such that the

35 compound is synthesized in the subject and the subject is treated for a disorder associated with β-amyloidosis. Preferably, the disorder is Alzheimer's disease.

Brief Description of the Drawing   5

Figure 1 is a graphic representation of the turbidity of a β-AP$_{1-40}$ solution, as measured by optical density at 400 nm, either in the absence of a β-amyloid modulator or in the presence of the β-amyloid modulator N-biotinyl-βAP$_{1-40}$

5   (1 %, or 5%).

Figure 2 is a schematic representation of compounds which can be used to modify a β-AP or an Aβ aggregation core domain to form a β-amyloid modulator of the invention.

Figure 3 is a graphic representation of the toxicity of Aβ$_{1-40}$ aggregates, but not Aβ$_{1-40}$ monomers, to cultured neuronal cells.

10   Figure 4 is a graphic representation of the aggregation of Aβ$_{1-40}$ in the presence of an equimolar amount of cholyl-Aβ$_{6-20}$ (panel A), a ~2-fold molar excess of cholyl-Aβ$_{6-20}$ (panel B) or a ~6-fold molar excess of cholyl-Aβ$_{6-20}$ (panel C) and the corresponding toxicity of the aggregates of panels A. B and C to cultured neuronal cells (panels D. E and F, respectively).

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Detailed Description of the Invention

This invention pertains to compounds. and pharmaceutical compositions thereof, that can modulate the aggregation of amyloidogenic proteins and peptides. in particular compounds that can modulate the aggregation of natural β amyloid peptides (β-AP) and

20   inhibit the neurotoxicity of natural β-APs. A compound of the invention that modulates aggregation of natural β-AP, referred to herein interchangeably as a β amyloid modulator compound. a β amyloid modulator or simply a modulator. alters the aggregation of natural β-AP when the modulator is contacted with natural β-AP. Thus. a compound of the invention acts to alter the natural aggregation process or rate for β-AP. thereby disrupting this process.

25   Preferably. the compounds inhibit β-AP aggregation. Furthermore. the invention provides subregions of the β amyloid peptide that are sufficient. when appropriately modified as described herein. to alter (and preferably inhibit) aggregation of natural β amyloid peptides when contacted with the natural β amyloid peptides. In particular. preferred modulator compounds of the invention are comprised of a modified form of an Aβ aggregation core

30   domain. modeled after the aforementioned Aβ subregion (as described further below). which is sufficient to alter (and preferably inhibit) the natural aggregation process or rate for β-AP. This Aβ aggregation core domain can comprises as few as three amino acid residues (or derivative. analogues or mimetics thereof). Moreover. while the amino acid sequence of the Aβ aggregation core domain can directly correspond to an amino acid sequence found in

35   natural β-AP. it is not essential that the amino acid sequence directly correspond to a β-AP sequence. Rather, amino acid residues derived from a preferred subregion of β-AP (a hydrophobic region centered around positions 17-20) can be rearranged in order and/or substituted with homologous residues within a modulator compound of the invention and yet maintain their inhibitory activity (described further below).

9

The β amyloid modulator compounds of the invention can be selected based upon their ability to inhibit the aggregation of natural β-AP *in vitro* and/or inhibit the neurotoxicity of natural β-AP fibrils for cultured cells (using assays described herein). Accordingly, the preferred modulator compounds inhibit the aggregation of natural β-AP and/or inhibit the

5   neurotoxicity of natural β-AP. However, modulator compounds selected based on one or both of these properties may have additional properties *in vivo* that may be beneficial in the treatment of amyloidosis. For example, the modulator compound may interfere with processing of natural β-AP (either by direct or indirect protease inhibition) or by modulation of processes that produce toxic β-AP, or other APP fragments, *in vivo*. Alternatively,

10   modulator compounds may be selected based on these latter properties. rather than inhibition of Aβ aggregation *in vitro*. Moreover. modulator compounds of the invention that are selected based upon their interaction with natural β-AP also may interact with APP or with other APP fragments.

As used herein. a "modulator" of β-amyloid aggregation is intended to refer to an

15   agent that, when contacted with natural β amyloid peptides. alters the aggregation of the natural β amyloid peptides. The term "aggregation of β amyloid peptides" refers to a process whereby the peptides associate with each other to form a multimeric. largely insoluble complex. The term "aggregation" further is intended to encompass β amyloid fibril formation and also encompasses β–amyloid plaques.

20   The terms "natural β-amyloid peptide", "natural β-AP" and "natural Aβ peptide", used interchangeably herein. are intended to encompass naturally occurring proteolytic cleavage products of the β amyloid precursor protein (APP) which are involved in β-AP aggregation and β-amyloidosis. These natural peptides include β-amyloid peptides having 39-43 amino acids (*i.e.*, $A\beta_{1-39}$, $A\beta_{1-40}$, $A\beta_{1-41}$, $A\beta_{1-42}$ and $A\beta_{1-43}$). The amino-terminal amino acid

25   residue of natural β-AP corresponds to the aspartic acid residue at position 672 of the 770 amino acid residue form of the amyloid precursor protein ("APP-770"). The 43 amino acid long form of natural β-AP has the amino acid sequence

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT

(also shown in SEQ ID NO: 1), whereas the shorter forms have 1-4 amino acid residues

30   deleted from the carboxy-terminal end. The amino acid sequence of APP-770 from position 672 (*i.e.*, the amino-terminus of natural β-AP) to its C-terminal end (103 amino acids) is shown in SEQ ID NO: 2. The preferred form of natural β-AP for use in the aggregation assays described herein is $A\beta_{1-40}$.

In the presence of a modulator of the invention. aggregation of natural β amyloid

35   peptides is "altered" or "modulated". The various forms of the term "alteration" or "modulation" are intended to encompass both inhibition of β-AP aggregation and promotion of β-AP aggregation. Aggregation of natural β-AP is "inhibited" in the presence of the modulator when there is a decrease in the amount and/or rate of β-AP aggregation as compared to the amount and/or rate of β-AP aggregation in the absence of the modulator.

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The various forms of the term "inhibition" are intended to include both complete and partial inhibition of β-AP aggregation. Inhibition of aggregation can be quantitated as the fold increase in the lag time for aggregation or as the decrease in the overall plateau level of aggregation (i.e., total amount of aggregation), using an aggregation assay as described in the

5   Examples. In various embodiments, a modulator of the invention increases the lag time of aggregation at least 1.2-fold, 1.5-fold, 1.8-fold, 2-fold, 2.5-fold, 3-fold, 4-fold or 5-fold. In various other embodiments, a modulator of the invention inhibits the plateau level of aggregation at least 10%, 20%, 30%, 40 %, 50 %, 75 % or 100 %.

A modulator which inhibits β-AP aggregation (an "inhibitory modulator compound")

10 can be used to prevent or delay the onset of β-amyloid deposition. Moreover, as demonstrated in Example 10, inhibitory modulator compounds of the invention inhibit the formation and/or activity of neurotoxic aggregates of natural Aβ peptide (i.e., the inhibitory compounds can be used to inhibit the neurotoxicity of β-AP). Still further, also as demonstrated in Example 10, the inhibitory compounds of the invention can be used to

15 reduce the neurotoxicity of preformed β-AP aggregates, indicating that the inhibitory modulators can either bind to preformed Aβ fibrils or soluble aggregate and modulate their inherent neurotoxicity or that the modulators can perturb the equilibrium between monomeric and aggregated forms of β-AP in favor of the non-neurotoxic form.

Alternatively, in another embodiment, a modulator compound of the invention

20 promotes the aggregation of natural Aβ peptides. The various forms of the term "promotion" refer to an increase in the amount and/or rate of β-AP aggregation in the presence of the modulator, as compared to the amount and/or rate of β-AP aggregation in the absence of the modulator. Such a compound which promotes Aβ aggregation is referred to as a stimulatory modulator compound. Stimulatory modulator compounds may be useful for sequestering β-

25 amyloid peptides, for example in a biological compartment where aggregation of β-AP may not be deleterious to thereby deplete β-AP from a biological compartment where aggregation of β-AP is deleterious. Moreover, stimulatory modulator compounds can be used to promote Aβ aggregation in in vitro aggregation assays (e.g., assays such as those described in the Examples), for example in screening assays for test compounds that can then inhibit or

30 reverse this Aβ aggregation (i.e., a stimulatory modulator compound can act as a "seed" to promote the formation of Aβ aggregates).

In a preferred embodiment, the modulators of the invention are capable of altering β-AP aggregation when contacted with a molar excess amount of natural β-AP. A "molar excess amount of natural β-AP" refers to a concentration of natural β-AP, in moles, that is

35 greater than the concentration, in moles, of the modulator. For example, if the modulator and β-AP are both present at a concentration of 1 μM, they are said to be "equimolar", whereas if the modulator is present at a concentration of 1 μM and the β-AP is present at a concentration of 5 μM, the β-AP is said to be present at a 5-fold molar excess amount compared to the modulator. In preferred embodiments, a modulator of the invention is effective at altering

11

natural β-AP aggregation when the natural β-AP is present at at least a 2-fold, 3-fold or 5-fold molar excess compared to the concentration of the modulator. In other embodiments, the modulator is effective at altering β-AP aggregation when the natural β-AP is present at at least a 10-fold, 20-fold, 33-fold, 50-fold, 100-fold, 500-fold or 1000-fold molar excess compared to the concentration of the modulator.

Various additional aspects of the modulators of the invention, and the uses thereof, are described in further detail in the following subsections.

I. Modulator Compounds

In one embodiment, a modulator of the invention comprises a β-amyloid peptide compound comprising the formula:

$$(Xaa)\!\!\!\nearrow^{An}$$

wherein Xaa is a β-amyloid peptide. A is a modulating group attached directly or indirectly to the β-amyloid peptide of the compound such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. and n is an integer selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

Preferably, β-amyloid peptide of the compound has an amino-terminal amino acid residue corresponding to position 668 of β-amyloid precursor protein-770 (APP-770) or to a residue carboxy-terminal to position 668 of APP-770. The amino acid sequence of APP-770 from position 668 to position 770 (*i.e.*, the carboxy terminus) is shown below and in SEQ ID NO: 2:

EVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVITL
VMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN

More preferably, the amino-terminal amino acid residue of the β-amyloid peptide corresponds to position 672 of APP-770 (position 5 of the amino acid sequence of SEQ ID NO: 2) or to a residue carboxy-terminal to position 672 of APP-770. Although the β-amyloid peptide of the compound may encompass the 103 amino acid residues corresponding to positions 668-770 of APP-770, preferably the peptide is between 6 and 60 amino acids in length, more preferably between 10 and 43 amino acids in length and even more preferably between 10 and 25 amino acid residues in length.

As used herein, the term "β amyloid peptide", as used in a modulator of the invention is intended to encompass peptides having an amino acid sequence identical to that of the natural sequence in APP, as well as peptides having acceptable amino acid substitutions from the natural sequence. Acceptable amino acid substitutions are those that do not affect the

12

ability of the peptide to alter natural β-AP aggregation. Moreover, particular amino acid
substitutions may further contribute to the ability of the peptide to alter natural β-AP
aggregation and/or may confer additional beneficial properties on the peptide (e.g., increased
solubility, reduced association with other amyloid proteins, etc.). For example, substitution
5   of hydrophobic amino acid residues for the two phenylalanine residues at positions 19 and 20
of natural β-AP (positions 19 and 20 of the amino acid sequence shown in SEQ ID NO: 1)
may further contribute to the ability of the peptide to alter β-AP aggregation (see Hilbich, C.
(1992) J. Mol. Biol. 228:460-473). Thus, in one embodiment, the β-AP of the compound
consists of the amino acid sequence shown below and in SEQ ID NO: 3:

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DAEFRHDSGYEVHHQKLV(Xaa$_{19}$)(Xaa$_{20}$)AEDVGSNKGAIIGLMVGGVVIAT

(or an amino-terminal or carboxy-terminal deletion thereof), wherein Xaa is a hydrophobic
amino acid. Examples of hydrophobic amino acids are isoleucine, leucine, threonine, serine,
15   alanine, valine or glycine. Preferably, $F_{19}F_{20}$ is substituted with $T_{19}T_{20}$ or $G_{19}I_{20}$.
        Other suitable amino acid substitutions include replacement of amino acids in the
human peptide with the corresponding amino acids of the rodent β-AP peptide. The three
amino acid residues that differ between human and rat β-AP are at positions 5, 10 and 13 of
the amino acid sequence shown in SEQ ID NOs: 1 and 3. A human β-AP having the human
20   to rodent substitutions Arg$_5$ to Gly, Tyr$_{10}$ to Phe and His$_{13}$ to Arg has been shown to retain
the properties of the human peptide (see Fraser, P.E. et al. (1992) Biochemistry 31:10716-
10723; and Hilbich, C. et al. (1991) Eur. J. Biochem. 201:61-69). Accordingly, a human β-
AP having rodent β-AP a.a. substitutions is suitable for use in a modulator of the invention.
        Other possible β-AP amino acid substitutions are described in Hilbich, C. et al.
25   (1991) J. Mol. Biol. 218:149-163; and Hilbich, C. (1992) J. Mol. Biol. 228:460-473.
Moreover, amino acid substitutions that affect the ability of β-AP to associate with other
proteins can be introduced. For example, one or more amino acid substitutions that reduce
the ability of β-AP to associate with the serpin enzyme complex (SEC) receptor, α1-
antichymotrypsin (ACT) and/or apolipoprotein E (ApoE) can be introduced. A preferred
30   substitution for reducing binding to the SEC receptor is $L_{34}M_{35}$ to $A_{34}A_{35}$ (at positions 34
and 35 of the amino acid sequences shown in SEQ ID NOs: 1 and 3). A preferred
substitution for reducing binding to ACT is S_8 to A_8 (at position 8 of the amino acid
sequences shown in SEQ ID NOs: 1 and 3).
        Alternative to β-AP amino acid substitutions described herein or known in the art, a
35   modulator composed, at least in part, of an amino acid-substituted β amyloid peptide can be
prepared by standard techniques and tested for the ability to alter β-AP aggregation using an
aggregation assay described herein. To retain the properties of the original modulator,
preferably conservative amino acid substitutions are made at one or more amino acid
residues. A "conservative amino acid substitution" is one in which the amino acid residue is

13

replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, 5   cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), β-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Accordingly, a modulator composed of a β amyloid peptide having an amino acid sequence that is mutated from that of the wild-type sequence in APP-770 yet which still retains the 10   ability to alter natural β-AP aggregation is within the scope of the invention.

As used herein, the term "β amyloid peptide" is further intended to include peptide analogues or peptide derivatives or peptidomimetics that retain the ability to alter natural β-AP aggregation as described herein. For example, a β amyloid peptide of a modulator of the invention may be modified to increase its stability, bioavailability, solubility, etc. The terms 15   "peptide analogue", "peptide derivative" and "peptidomimetic" as used herein are intended to include molecules which mimic the chemical structure of a peptide and retain the functional properties of the peptide. Approaches to designing peptide analogs are known in the art. For example, see Farmer, P.S. in Drug Design (E.J. Ariens, ed.) Academic Press, New York, 1980, vol. 10, pp. 119-143; Ball. J.B. and Alewood, P.F. (1990) *J. Mol. Recognition* 3:55; 20   Morgan, B.A. and Gainor, J.A. (1989) *Ann. Rep. Med. Chem.* 24:243; and Freidinger, R.M. (1989) *Trends Pharmacol. Sci.* 10:270. Examples of peptide analogues, derivatives and peptidomimetics include peptides substituted with one or more benzodiazepine molecules (see *e.g.*, James. G.L. *et al.* (1993) *Science* 260:1937-1942), peptides with methylated amide linkages and "retro-inverso" peptides (see U.S. Patent No. 4,522,752 by Sisto). Peptide 25   analogues, peptide derivatives and peptidomimetic are described in further detail below with regard to compounds comprising an Aβ aggregation core domain.

In a modulator of the invention having the formula shown above, a modulating group ("A") is attached directly or indirectly to the β-amyloid peptide of the modulator (As used herein, the term "modulating group" and "modifying group" are used interchangeably to 30   describe a chemical group directly or indirectly attached to an Aβ derived peptidic structure). For example, the modulating group can be directly attached by covalent coupling to the β-amyloid peptide or the modulating group can be attached indirectly by a stable non-covalent association. In one embodiment of the invention, the modulating group is attached to the amino-terminus of the β-amyloid peptide of the modulator. Accordingly, the modulator can 35   comprise a compound having a formula:

$$A-\overset{H}{N}-(\ Xaa\ )$$

14

Alternatively, in another embodiment of the invention, the modulating group is attached to the carboxy-terminus of the β-amyloid peptide of the modulator. Accordingly, the modulator can comprise a compound having a formula:

$$(Xaa) \underset{}{\overset{O}{\underset{\|}{-C-}}} A$$

In yet another embodiment, the modulating group is attached to the side chain of at least one amino acid residues of the β-amyloid peptide of the compound (e.g., through the epsilon amino group of a lysyl residue(s), through the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain).

The modulating group is selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Accordingly, since the β-AP peptide of the compound is modified from its natural state, the modulating group "A" as used herein is not intended to include hydrogen. In a preferred embodiment, the modulating group is a biotin compound of the formula:

wherein X_1-X_3 are each independently selected from the group consisting of S, O and NR_2, wherein R_2 is hydrogen, or an aryl, lower alkyl, alkenyl or alkynyl moiety; W is $=O$ or NR_2; R_1 is a lower alkylenyl moiety and Y is a direct bond or a spacer molecule selected for its ability to react with a target group on a β-AP. At least one of X_1-X_3 or W is an NR_2 group.

The term "aryl" is intended to include aromatic moieties containing substituted or unsubstituted ring(s), e.g., benzyl, napthyl, etc. Other more complex fused ring moieties also are intended to be included.

The term "lower alkyl or alkylenyl moiety" refers to a saturated, straight or branched chain (or combination thereof) hydrocarbon containing 1 to about 6 carbon atoms, more preferably from 1 to 3 carbon atoms. The terms "lower alkenyl moiety" and "lower alkynyl moiety" refer to unsaturated hydrocarbons containing 1 to about 6 carbon atoms, more preferably 1 to 3 carbon atoms. Preferably, R_2 contains 1 to 3 carbon atoms. Preferably, R_1 contains 4 carbon atoms.

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The spacer molecule (Y) can be, for example, a lower alkyl group or a linker peptide, and is preferably selected for its ability to link with a free amino group (e.g., the α-amino group at the amino-terminus of a β-AP). Thus, in a preferred embodiment, the biotin compound modifies the amino-terminus of a β-amyloid peptide.

5   Additional suitable modulating groups may include other cyclic and heterocyclic compounds and other compounds having similar steric "bulk". Non-limiting examples of compounds which can be used to modify a β-AP are shown schematically in Figure 2, and include N-acetylneuraminic acid, cholic acid, trans-4-cotininecarboxylic acid, 2-imino-1-imidazolidineacetic acid, (S)-(-)-indoline-2-carboxylic acid, (-)-menthoxyacetic acid, 2-

10   norbornaneacetic acid, γ-oxo-5-acenaphthenebutyric acid, (-)-2-oxo-4-thiazolidinecarboxylic acid, tetrahydro-3-furoic acid, 2-iminobiotin-N-hydroxysuccinimide ester, diethylenetriaminepentaacetic dianhydride, 4-morpholinecarbonyl chloride, 2-thiopheneacetyl chloride, 2-thiophenesulfonyl chloride, 5-(and 6-)-carboxyfluorescein (succinimidyl ester), fluorescein isothiocyanate, and acetic acid (or derivatives thereof).

15   Suitable modulating groups are described further in subsection II below.

In a modulator of the invention, a single modulating group may be attached to a β-amyloid peptide (e.g., n=1 in the formula shown above) or multiple modulating groups may be attached to the peptide. The number of modulating groups is selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural

20   β-amyloid peptides. However, n preferably is an integer between 1 and 60, more preferably between 1 and 30 and even more preferably between 1 and 10 or 1 and 5.

In another embodiment, a β-amyloid modulator compound of the invention comprises an Aβ aggregation core domain (abbreviated as ACD) coupled directly or indirectly to a

25   modifying group such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. As used herein, an "Aβ aggregation core domain" is intended to refer to a structure that is modeled after a subregion of a natural β-amyloid peptide which is sufficient to modulate aggregation of natural β-APs when this subregion of the natural β-AP is

30   appropriately modified as described herein (e.g., modified at the amino-terminus). The term "subregion of a natural β-amyloid peptide" is intended to include amino-terminal and/or carboxy-terminal deletions of natural β-AP. The term "subregion of natural β-AP" is not intended to include full-length natural β-AP (i.e., "subregion" does not include $A\beta_{1-39}$, $A\beta_{1-40}$, $A\beta_{1-41}$, $A\beta_{1-42}$ and $A\beta_{1-43}$).

35   Although not intending to be limited by mechanism, the ACD of the modulators of the invention is thought to confer a specific targeting function on the compound that allows the compound to recognize and specifically interact with natural β-AP. Preferably, the ACD is modeled after a subregion of natural β-AP that is less than 15 amino acids in length and more preferably is between 3-10 amino acids in length. In various embodiments, the ACD is

10

modeled after a subregion of β-AP that is 10, 9, 8, 7, 6, 5, 4 or 3 amino acids in length. In one embodiment, the subregion of β-AP upon which the ACD is modeled is an internal or carboxy-terminal region of β-AP (*i.e.*, downstream of the amino-terminus at amino acid position 1). In another embodiment, the ACD is modeled after a subregion of β-AP that is

5   hydrophobic. In certain specific embodiments, the term Aβ aggregation core domain specifically excludes β-AP subregions corresponding to amino acid positions 1-15 (Aβ$_{1-15}$), 6-20 (Aβ$_{6-20}$) and 16-40 (Aβ$_{16-40}$).

An Aβ aggregation core domain can be comprised of amino acid residues linked by peptide bonds. That is, the ACD can be a peptide corresponding to a subregion of β-AP.

10   Alternatively, an Aβ aggregation core domain can be modeled after the natural Aβ peptide region but may be comprised of a peptide analogue, peptide derivative or peptidomimetic compound. or other similar compounds which mimics the structure and function of the natural peptide. Accordingly, as used herein, an "Aβ aggregation core domain" is intended to include peptides. peptide analogues, peptide derivatives and peptidomimetic compounds

15   which. when appropriately modified. retain the aggregation modulatory activity of the modified natural Aβ peptide subregion. Such structures that are designed based upon the amino acid sequence are referred to herein as "Aβ derived peptidic structures." Approaches to designing peptide analogues, derivatives and mimetics are known in the art. For example, see Farmer, P.S. in Drug Design (E.J. Ariens, ed.) Academic Press. New York. 1980, vol. 10,

20   pp. 119-143; Ball. J.B. and Alewood. P.F. (1990) *J. Mol. Recognition* 3:55; Morgan. B.A. and Gainor. J.A. (1989) *Ann. Rep. Med. Chem.* 24:243; and Freidinger, R.M. (1989) *Trends Pharmacol. Sci.* 10:270. See also Sawyer. T.K. (1995) "Peptidomimetic Design and Chemical Approaches to Peptide Metabolism" in Taylor, M.D. and Amidon. G.L. (eds.) Peptide-Based Drug Design: Controlling Transport and Metabolism. Chapter 17: Smith. A.B.

25   3rd. *et al.* (1995) *J. Am. Chem. Soc.* 117:11113-11123: Smith. A.B. 3rd. *et al.* (1994) *J. Am. Chem. Soc.* 116:9947-9962; and Hirschman. R.. *et al.* (1993) *J. Am. Chem. Soc.* 115:12550-12568.

As used herein. a "derivative" of a compound X (*e.g.*, a peptide or amino acid) refers to a form of X in which one or more reaction groups on the compound have been derivatized

30   with a substituent group. Examples of peptide derivatives include peptides in which an amino acid side chain. the peptide backbone. or the amino- or carboxy-terminus has been derivatized (*e.g.*, peptidic compounds with methylated amide linkages). As used herein an "analogue" of a compound X refers to a compound which retains chemical structures of X necessary for functional activity of X yet which also contains certain chemical structures

35   which differ from X. An examples of an analogue of a naturally-occurring peptide is a peptides which includes one or more non-naturally-occurring amino acids. As used herein, a "mimetic" of a compound X refers to a compound in which chemical structures of X necessary for functional activity of X have been replaced with other chemical structures which mimic the conformation of X. Examples of peptidomimetics include peptidic

17

compounds in which the peptide backbone is substituted with one or more benzodiazepine molecules (see e.g., James, G.L. et al. (1993) *Science* 260:1937-1942), peptides in which all L-amino acids are substituted with the corresponding D-amino acids and "retro-inverso" peptides (see U.S. Patent No. 4,522,752 by Sisto), described further below.

5   The term mimetic, and in particular, peptidomimetic, is intended to include isosteres. The term "isostere" as used herein is intended to include a chemical structure that can be substituted for a second chemical structure because the steric conformation of the first structure fits a binding site specific for the second structure. The term specifically includes peptide back-bone modifications (i.e., amide bond mimetics) well known to those skilled in

10   the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. Several peptide backbone modifications are known, including ψ[CH$_2$S], ψ [CH$_2$NH], ψ[CSNH$_2$], ψ[NHCO], ψ[COCH$_2$], and ψ[(E) or (Z) CH=CH]. In the nomenclature used above, ψ indicates the absence of an amide bond. The structure that

15   replaces the amide group is specified within the brackets. Other examples of isosteres include peptides substituted with one or more benzodiazepine molecules (see e.g., James, G.L. et al. (1993) *Science* 260:1937-1942)

Other possible modifications include an N-alkyl (or aryl) substitution (ψ[CONR]), backbone crosslinking to construct lactams and other cyclic structures, substitution of all D-

20   amino acids for all L-amino acids within the compound ("inverso" compounds) or retro-inverso amino acid incorporation (ψ[NHCO]). By "inverso" is meant replacing L-amino acids of a sequence with D-amino acids, and by "retro-inverso" or "enantio-retro" is meant reversing the sequence of the amino acids ("retro") and replacing the L-amino acids with D-amino acids. For example, if the parent peptide is Thr-Ala-Tyr, the retro modified form is

25   Tyr-Ala-Thr, the inverso form is thr-ala-tyr, and the retro-inverso form is tyr-ala-thr (lower case letters refer to D-amino acids). Compared to the parent peptide, a retro-inverso peptide has a reversed backbone while retaining substantially the original spatial conformation of the side chains, resulting in a retro-inverso isomer with a topology that closely resembles the parent peptide. See Goodman *et al.* "*Perspectives in Peptide Chemistry*" pp. 283-294

30   (1981). See also U.S. Patent No. 4,522,752 by Sisto for further description of "retro-inverso" peptides.

Other derivatives of the modulator compounds of the invention include C-terminal hydroxymethyl derivatives, O-modified derivatives (e.g., C-terminal hydroxymethyl benzyl ether), N-terminally modified derivatives including substituted amides such as alkylamides

35   and hydrazides and compounds in which a C-terminal phenylalanine residue is replaced with a phenethylamide analogue (e.g., Val-Phe-phenethylamide as an analogue of the tripeptide Val-Phe-Phe).

In a preferred embodiment, the ACD of the modulator is modeled after the subregion of β-AP encompassing amino acid positions 17-20 (i.e., Leu-Val-Phe-Phe; SEQ ID NO: 12).

14

As described further in Examples 7, 8 and 9, peptide subregions of $A\beta_{1-40}$ were prepared, amino-terminally modified and evaluated for their ability to modulate aggregation of natural β-amyloid peptides. One subregion that was effective at inhibiting aggregation was $A\beta_{6-20}$ (*i.e.*, amino acid residues 6-20 of the natural $A\beta_{1-40}$ peptide, the amino acid sequence of

5 which is shown in SEQ ID NO: 4). Amino acid residues were serially deleted from the amino-terminus or carboxy terminus of this subregion to further delineate a minimal subregion that was sufficient for aggregation inhibitory activity. This process defined $A\beta_{17-20}$ (*i.e.*, amino acid residues 17-20 of the natural $A\beta_{1-40}$ peptide) as a minimal subregion that, when appropriately modified, is sufficient for aggregation inhibitory activity.

10 Accordingly, an "Aβ aggregation core domain" within a modulator compound of the invention can be modeled after $A\beta_{17-20}$. In one embodiment, the Aβ aggregation core domain comprises $A\beta_{17-20}$ itself (*i.e.*, a peptide comprising the amino acid sequence leucine-valine-phenylalanine-phenylalanine; SEQ ID NO: 12). In other embodiments, the structure of $A\beta_{17-20}$ is used as a model to design an Aβ aggregation core domain having similar

15 structure and function to $A\beta_{17-20}$. For example, peptidomimetics, derivatives or analogues of $A\beta_{17-20}$ (as described above) can be used as an Aβ aggregation core domain. In addition to $A\beta_{17-20}$, the natural Aβ peptide is likely to contain other minimal subregions that are sufficient for aggregation inhibitory activity. Such additional minimal subregions can be identified by the processes described in Examples 7, 8 and 9, wherein a 15mer subregion of

20 $A\beta_{1-40}$ is serially deleted from the amino-terminus or carboxy terminus, the deleted peptides are appropriately modified and then evaluated for aggregation inhibitory activity.

One form of the β-amyloid modulator compound comprising an Aβ aggregation core domain modeled after $A\beta_{17-20}$ coupled directly or indirectly to at least one modifying group has the formula:

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$$(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}Z)_n^{An}$$

wherein Xaa_1 and Xaa_3 are amino acid structures;

Xaa_2 is a valine structure;

Xaa_4 is a phenylalanine structure;

30 Y, which may or may not be present, is a peptidic structure having the formula $(Xaa)_a$, wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

Z, which may or may not be present, is a peptidic structure having the formula $(Xaa)_b$, wherein Xaa is any amino acid structure and b is an integer from 1 to 15; and

A is a modifying group attached directly or indirectly to the compound and n

35 is an integer;

Xaa_1, Xaa_3, Y, Z, A and n being selected such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

19

Preferably, a modulator compound of the above formula inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides and/or inhibits Aβ neurotoxicity. Alternatively, the modulator compound can promote aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. The type

5   and number of modifying groups ("A") coupled to the modulator are selected such that the compound alters (and preferably inhibits) aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. A single modifying group can be coupled to the modulator (i.e., n=1 in the above formula) or, alternatively, multiple modifying groups can be coupled to the modulator. In various embodiments, n is an integer between 1 and 60,

10   between 1 and 30, between 1 and 10, between 1 and 5 or between 1 and 3. Suitable types of modifying groups are described further in subsection II below.

As demonstrated in Example 9, amino acid positions 18 (Val_{18}) and 20 (Phe_{20}) of $Aβ_{17-20}$ (corresponding to Xaa_2 and Xaa_4) are particularly important within the core domain for inhibitory activity of the modulator compound. Accordingly, these positions are

15   conserved within the core domain in the formula shown above. The terms "valine structure" and "phenylalanine structure" as used in the above formula are intended to include the natural amino acids, as well as non-naturally-occurring analogues, derivatives and mimetics of valine and phenylalanine, respectively, (including D-amino acids) which maintain the functional activity of the compound. Moreover, although Val_{18} and Phe_{20} have an important functional

20   role, it is possible that Xaa_2 and/or Xaa_4 can be substituted with other naturally-occurring amino acids that are structurally related to valine or phenylalanine, respectively, while still maintaining the activity of the compound. Thus, the terms "valine structure" is intended to include conservative amino acid substitutions that retain the activity of valine at Xaa_2, and the term "phenylalanine structure" is intended to include conservative amino acid

25   substitutions that retain the activity of phenylalanine at Xaa_4. However, the term "valine structure" is not intended to include threonine.

In contrast to positions 18 and 20 of $Aβ_{17-20}$, a Phe to Ala substitution at position 19 (corresponding to Xaa_3) did not abolish the activity of the modulator, indicating position 19 may be more amenable to amino acid substitution. In various embodiments of the above

30   formula, positions Xaa_1 and Xaa_3 are any amino acid structure. The term "amino acid structure" is intended to include natural and non-natural amino acids as well as analogues, derivatives and mimetics thereof, including D-amino acids. In a preferred embodiment of the above formula, Xaa_1 is a leucine structure and Xaa_3 is a phenylalanine structure (i.e., modeled after Leu_{17} and Phe_{19}, respectively, in the natural Aβ peptide sequence). The term

35   "leucine structure" is used in the same manner as valine structure and phenylalanine structure described above. Alternatively, an another embodiment, Xaa_3 is an alanine structure.

The four amino acid structure ACD of the modulator of the above formula can be flanked at the amino-terminal side, carboxy-terminal side, or both, by peptidic structures derived either from the natural Aβ peptide sequence or from non-Aβ sequences. The term

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"peptidic structure" is intended to include peptide analogues, derivatives and mimetics thereof, as described above. The peptidic structure is composed of one or more linked amino acid structures, the type and number of which in the above formula are variable. For example, in one embodiment, no additional amino acid structures flank the Xaa_1-Xaa_2-Xaa_3-

5   Xaa_4 core sequence (i.e., Y and Z are absent in the above formula). In another embodiment, one or more additional amino acid structures flank only the amino-terminus of the core sequences (i.e., Y is present but Z is absent in the above formula). In yet another embodiment, one or more additional amino acid structures flank only the carboxy-terminus of the core sequences (i.e., Z is present but Y is absent in the above formula). The length of

10   flanking Z or Y sequences also is variable. For example, in one embodiment, a and b are integers from 1 to 15. More preferably, a and b are integers between 1 and 10. Even more preferably, a and b are integers between 1 and 5. Most preferably, a and b are integers between 1 and 3.

One form of the β-amyloid modulator compound comprising an Aβ aggregation core

15   domain modeled after $Aβ_{17-20}$ coupled directly or indirectly to at least one modifying group has the formula:

$$A\text{-}(Y)\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}(Z)\text{-}B$$

wherein Xaa_1 and Xaa_3 are amino acids or amino acid mimetics;

20   Xaa_2 is valine or a valine mimetic

Xaa_4 is phenylalanine or a phenylalanine mimetic;

Y, which may or may not be present, is a peptide or peptidomimetic having the formula $(Xaa)_a$, wherein Xaa is any amino acid or amino acid mimetic and a is an integer from 1 to 15;

25   Z, which may or may not be present, is a peptide or peptidomimetic having the formula $(Xaa)_b$, wherein Xaa is any amino acid or amino acid mimetic and b is an integer from 1 to 15; and

A and B, at least one of which is present, are modifying groups attached directly or indirectly to the amino terminus and carboxy terminus, respectively, of the

30   compound;

Xaa_1, Xaa_3, Y, Z, A and B being selected such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

In this embodiment, the modulator compound is specifically modified at either its

35   amino-terminus, its carboxy-terminus, or both. The terminology used in this formula is the same as described above. Suitable modifying groups are described in subsection II below. In one embodiment, the compound is modified only at its amino terminus (i.e., B is absent and the compound comprises the formula: $A\text{-}(Y)\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}(Z)$). In another embodiment, the compound is modified only at its carboxy-terminus (i.e., A is absent and the

21

compound comprises the formula: (Y)-Xaa$_1$-Xaa$_2$-Xaa$_3$-Xaa$_4$-(Z)-B). In yet another
embodiment, the compound is modified at both its amino- and carboxy termini (*i.e.*, the
compound comprises the formula: A-(Y)-Xaa$_1$-Xaa$_2$-Xaa$_3$-Xaa$_4$-(Z)-B and both A and B are
present). As described above, the type and number of amino acid structures which flank the

5   Xaa$_1$-Xaa$_2$-Xaa$_3$-Xaa$_4$ core sequences in the above formula is variable. For example, in one
embodiment, a and b are integers from 1 to 15. More preferably, a and b are integers between
1 and 10. Even more preferably, a and b are integers between 1 and 5. Most preferably, a
and b are integers between 1 and 3.

           As demonstrated in Examples 7, 8 and 9, preferred Aβ modulator compounds of the

10   invention comprise modified forms of Aβ$_{14-21}$ (His-Gln-Lys-Leu-Val-Phe-Phe-Ala; SEQ ID
NO: 5), or amino-terminal or carboxy-terminal deletions thereof, with a preferred "minimal
core region" comprising Aβ$_{17-20}$. Accordingly, in specific embodiments. the invention
provides compounds comprising the formula:

15   A-Xaa$_1$-Xaa$_2$-Xaa$_3$-Xaa$_4$-Xaa$_5$-Xaa$_6$-Xaa$_7$-Xaa$_8$-B

           wherein Xaa1 is a histidine structure;
                   Xaa2 is a glutamine structure;
                   Xaa3 is a lysine structure;
20   Xaa4 is a leucine structure;
                   Xaa5 is a valine structure;
                   Xaa6 is a phenylalanine structure;
                   Xaa7 is a phenylalanine structure;
                   Xaa8 is an alanine structure;
25   A and B are modifying groups attached directly or indirectly to the amino
terminus and carboxy terminus, respectively, of the compound;
           and wherein Xaa$_1$-Xaa$_2$-Xaa$_3$, Xaa$_1$-Xaa$_2$ or Xaa$_1$ may or may not be present;
                   Xaa$_8$ may or may not be present; and
                   at least one of A and B is present.
30

           In one specific embodiment, the compound comprises the formula: A-Xaa$_4$-Xaa$_5$-
Xaa$_6$-Xaa$_7$-B (e.g, a modified form of Aβ$_{17-20}$, comprising an amino acid sequence Leu-Val-
Phe-Phe; SEQ ID NO: 12).
           In another specific embodiment, the compound comprises the formula: A-Xaa$_4$-

35   Xaa$_5$-Xaa$_6$-Xaa$_7$-Xaa$_8$-B (e.g, a modified form of Aβ$_{17-21}$, comprising an amino acid
sequence Leu-Val-Phe-Phe-Ala; SEQ ID NO: 11).
           In another specific embodiment, the compound comprises the formula: A-Xaa$_3$-
Xaa$_4$-Xaa$_5$-Xaa$_6$-Xaa$_7$-B (*e.g.*, a modified form of Aβ$_{16-20}$, comprising an amino acid
sequence Lys-Leu-Val-Phe-Phe; SEQ ID NO: 10).

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In another specific embodiment, the compound comprises the formula: A-Xaa₃- [A-Xaa_3-]

In another specific embodiment, the compound comprises the formula: A-Xaa_3-Xaa_4-Xaa_5-Xaa_6-Xaa_7-Xaa_8-B (*e.g.*, a modified form of Aβ_{16-21}, comprising an amino acid sequence Lys-Leu-Val-Phe-Phe-Ala; SEQ ID NO: 9).

In another specific embodiment, the compound comprises the formula: A-Xaa_2-Xaa_3-Xaa_4-Xaa_5-Xaa_6-Xaa_7-B (*e.g.*, a modified form of Aβ_{15-20}, comprising an amino acid sequence Gln-Lys-Leu-Val-Phe-Phe; SEQ ID NO: 8).

In another specific embodiment, the compound comprises the formula: A-Xaa_2-Xaa_3-Xaa_4-Xaa_5-Xaa_6-Xaa_7-Xaa_8-B (*e.g.*, a modified form of Aβ_{15-21}, comprising an amino acid sequence Gln-Lys-Leu-Val-Phe-Phe-Ala; SEQ ID NO: 7).

In another specific embodiment, the compound comprises the formula: A-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Xaa_5-Xaa_6-Xaa_7-B (*e.g.*, a modified form of Aβ_{14-20}, comprising an amino acid sequence His-Gln-Lys-Leu-Val-Phe-Phe; SEQ ID NO: 6).

In another specific embodiment, the compound comprises the formula: A-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Xaa_5-Xaa_6-Xaa_7-Xaa_8-B (*e.g.*, a modified form of Aβ_{14-21}, comprising an amino acid sequence His-Gln-Lys-Leu-Val-Phe-Phe-Ala; SEQ ID NO: 5).

In preferred embodiments of the aforementioned specific embodiments, A or B is a cholanoyl structure or a biotin-containing structure (described further in subsection II below).

In further experiments to delineate subregions of Aβ upon which an Aβ aggregation core domain can be modeled (the results of which are described in Example 11), it was demonstrated that a modulator compound having inhibitory activity can comprise as few as three Aβ amino acids residues (*e.g.*, Val-Phe-Phe, which corresponds to Aβ_{18-20} or Phe-Phe-Ala, which corresponds to Aβ_{19-21}). The results also demonstrated that a modulator compound having a modulating group at its carboxy-terminus is effective at inhibiting Aβ aggregation. Still further, the results demonstrated that the cholyl group, as a modulating group, can be manipulated while maintaining the inhibitory activity of the compounds and that an iodotyrosyl can be substituted for phenylalanine (*e.g.*, at position 19 or 20 of the Aβ sequence) while maintaining the ability of the compound to inhibit Aβ aggregation.

Still further, the results demonstrated that compounds with inhibitory activity can be created using amino acids residues that are derived from the Aβ sequence in the region of about positions 17-21 but wherein the amino acid sequence is rearranged or has a substitution with a non-Aβ-derived amino acid. Examples of such compounds include PPI-426, in which the sequence of Aβ_{17-21} (LVFFA) has been rearranged (FFVLA), PPI-372, in which the sequence of Aβ_{16-20} (KLVFF) has been rearranged (FKFVL), and PPI-388, -389 and -390, in which the sequence of Aβ_{17-21} (LVFFA) has been substituted at position 17, 18 or 19, respectively, with an alanine residue (AVFFA for PPI-388, LAFFA for PPI-389 and LVAFA for PPI-390). The inhibitory activity of these compounds indicate that the presence in the compound of an amino acid sequence directly corresponding to a portion of Aβ is not essential for inhibitory activity, but rather suggests that maintenance of the hydrophobic

23

nature of this core region, by inclusion of amino acid residues such as phenylalanine, valine, leucine, regardless of their precise order, can be sufficient for inhibition of Aβ aggregation. Accordingly, an Aβ aggregation core domain can be designed based on the direct Aβ amino acid sequence or can be designed based on a rearranged Aβ sequence which maintains the

5   hydrophobicity of the Aβ subregion, e.g., the region around positions 17-20. This region of Aβ contains the amino acid residues Leu, Val and Phe. Accordingly, preferred Aβ aggregation core domains are composed of at least three amino acid structures (as that term is defined hereinbefore, including amino acid derivatives, analogues and mimetics), wherein at least two of the amino acid structures are, independently, either a leucine structure, a valine

10   structure or a phenylalanine structure (as those terms are defined hereinbefore, including derivatives, analogues and mimetics).

         Thus, in another embodiment, the invention provides a β-amyloid modulator compound comprising a formula:

$$(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Z)_{A_n}$$

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         wherein Xaa_1, Xaa_2 and Xaa_3 are each amino acid structures and at least two of Xaa_1, Xaa_2 and Xaa_3 are, independently, selected from the group consisting of a leucine structure, a phenylalanine structure and a valine structure;

20   Y, which may or may not be present, is a peptidic structure having the formula $(Xaa)_a$, wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

                     Z, which may or may not be present, is a peptidic structure having the formula $(Xaa)_b$, wherein Xaa is any amino acid structure and b is an integer from 1 to 15; and

                     A is a modifying group attached directly or indirectly to the compound and n

25   is an integer;

         Xaa_1, Xaa_2, Xaa_3, Y, Z, A and n being selected such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

         Preferably, the compound inhibits aggregation of natural β-amyloid peptides when

30   contacted with the natural β-amyloid peptides. In preferred embodiments. Xaa_1 and Xaa_2 are each phenylalanine structures or Xaa_2 and Xaa_3 are each phenylalanine structures. "n" can be, for example, an integer between 1 and 5, whereas "a" and "b" can be, for example, integers between 1 and 5. The modifying group "A" preferably comprises a cyclic, heterocyclic or polycyclic group. More preferably, A contains a cis-decalin group, such as

35   cholanoyl structure or a cholyl group In other embodiments, A can comprise a biotin-containing group, a diethylene-triaminepentaacetyl group, a (-)-menthoxyacetyl group, a fluorescein-containing group or an N-acetylneuraminyl group. In yet other embodiments, the compound may promotes aggregation of natural β-amyloid peptides when contacted with the

24

natural β-amyloid peptides, may be further modified to alter a pharmacokinetic property of the compound or may be further modified to label the compound with a detectable substance.

In another embodiment, the invention provides a β-amyloid modulator compound comprising a formula:

$$A\text{-}(Y)\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}(Z)\text{-}B$$

wherein Xaa_1, Xaa_2 and Xaa_3 are each amino acid structures and at least two of Xaa_1, Xaa_2 and Xaa_3 are, independently, selected from the group consisting of a leucine structure, a phenylalanine structure and a valine structure;

Y, which may or may not be present, is a peptidic structure having the formula $(Xaa)_a$, wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

Z, which may or may not be present, is a peptidic structure having the formula $(Xaa)_b$, wherein Xaa is any amino acid structure and b is an integer from 1 to 15; and

A and B, at least one of which is present, are modifying groups attached directly or indirectly to the amino terminus and carboxy terminus, respectively, of the compound;

Xaa_1, Xaa_2, Xaa_3, Y, Z, A and B being selected such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

Preferably, the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. In preferred embodiments, Xaa_1 and Xaa_2 are each phenylalanine structures or Xaa_2 and Xaa_3 are each phenylalanine structures. In one subembodiment, the compound comprises the formula:

$$A\text{-}(Y)\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}(Z)$$

In another subembodiment, the compound comprises the formula:

$$(Y)\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}(Z)\text{-}B$$

"n" can be, for example, an integer between 1 and 5, whereas "a" and "b" can be, for example, integers between 1 and 5. The modifying group "A" preferably comprises a cyclic, heterocyclic or polycyclic group. More preferably, A contains a cis-decalin group, such as cholanoyl structure or a cholyl group In other embodiments, A can comprise a biotin-containing group, a diethylene-triaminepentaacetyl group, a (-)-menthoxyacetyl group, a fluorescein-containing group or an N-acetylneuraminyl group. In yet other embodiments, the compound may promote aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides, may be further modified to alter a pharmacokinetic property of the compound or may be further modified to label the compound with a detectable substance.

In preferred specific embodiments, the invention provides a β-amyloid modulator compound comprising a modifying group attached directly or indirectly to a peptidic structure, wherein the peptidic structure comprises amino acid structures having an amino

35

acid sequence selected from the group consisting of His-Gln-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 5), His-Gln-Lys-Leu-Val-Phe-Phe (SEQ ID NO: 6), Gln-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 7), Gln-Lys-Leu-Val-Phe-Phe (SEQ ID NO: 8), Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 9), Lys-Leu-Val-Phe-Phe (SEQ ID NO: 10), Leu-Val-Phe-Phe-Ala (SEQ ID
5   NO: 11), Leu-Val-Phe-Phe (SEQ ID NO: 12), Leu-Ala-Phe-Phe-Ala (SEQ ID NO: 13), Val-Phe-Phe (SEQ ID NO: 19), Phe-Phe-Ala (SEQ ID NO: 20), Phe-Phe-Val-Leu-Ala (SEQ ID NO: 21), Leu-Val-Phe-Phe-Lys (SEQ ID NO: 22), Leu-Val-Iodotyrosine-Phe-Ala (SEQ ID NO: 23), Val-Phe-Phe-Ala (SEQ ID NO: 24), Ala-Val-Phe-Phe-Ala (SEQ ID NO: 25), Leu-Val-Phe-Iodotyrosine-Ala (SEQ ID NO: 26), Leu-Val-Phe-Phe-Ala-Glu (SEQ ID NO: 27),
10   Phe-Phe-Val-Leu (SEQ ID NO: 28), Phe-Lys-Phe-Val-Leu (SEQ ID NO: 29), Lys-Leu-Val-Ala-Phe (SEQ ID NO: 30), Lys-Leu-Val-Phe-Phe-βAla (SEQ ID NO: 31) and Leu-Val-Phe-Phe-DAla (SEQ ID NO: 32).

These specific compounds can be further modified to alter a pharmacokinetic property of the compound and/or further modified to label the compound with a detectable substance.

15   The modulator compounds of the invention can be incorporated into pharmaceutical compositions (described further in subsection V below) and can be used in detection and treatment methods as described further in subsection VI below.

II. Modifying Groups

20   Within a modulator compound of the invention, a peptidic structure (such as an Aβ derived peptide, or an Aβ aggregation core domain, or an amino acid sequence corresponding to a rearranged Aβ aggregation core domain) is coupled directly or indirectly to at least one modifying group (abbreviated as MG). In one embodiment, a modulator compounds of the invention comprising an aggregation core domain coupled to a modifying group, the
25   compound can be illustrated schematically as MG-ACD. The term "modifying group" is intended to include structures that are directly attached to the peptidic structure (*e.g.*, by covalent coupling), as well as those that are indirectly attached to the peptidic structure (*e.g.*, by a stable non-covalent association or by covalent coupling to additional amino acid residues, or mimetics, analogues or derivatives thereof, which may flank the Aβ-derived
30   peptidic structure). For example, the modifying group can be coupled to the amino-terminus or carboxy-terminus of an Aβ-derived peptidic structure, or to a peptidic or peptidomimetic region flanking the core domain. Alternatively, the modifying group can be coupled to a side chain of at least one amino acid residue of an Aβ-derived peptidic structure, or to a peptidic or peptidomimetic region flanking the core domain (*e.g.*, through the epsilon amino group of
35   a lysyl residue(s), through the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain). Modifying groups covalently coupled to the peptidic structure can be attached by means and using methods well

34

known in the art for linking chemical structures, including, for example, amide, alkylamino, carbamate or urea bonds.

The term "modifying group" is intended to include groups that are not naturally coupled to natural Aβ peptides in their native form. Accordingly, the term "modifying group" is not intended to include hydrogen. The modifying group(s) is selected such that the modulator compound alters, and preferably inhibits, aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Although not intending to be limited by mechanism, the modifying group(s) of the modulator compounds of the invention is thought to function as a key pharmacophore which is important for conferring on the modulator the ability to disrupt Aβ polymerization.

In a preferred embodiment, the modifying group(s) comprises a cyclic, heterocyclic or polycyclic group. The term "cyclic group", as used herein, is intended to include cyclic saturated or unsaturated (i.e., aromatic) group having from about 3 to 10, preferably about 4 to 8, and more preferably about 5 to 7, carbon atoms. Exemplary cyclic groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cyclooctyl. Cyclic groups may be unsubstituted or substituted at one or more ring positions. Thus, a cyclic group may be substituted with, e.g., halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, heterocycles, hydroxyls, aminos, nitros, thiols amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, sulfonates, selenoethers, ketones, aldehydes, esters, $-CF_3$, $-CN$, or the like.

The term "heterocyclic group" is intended to include cyclic saturated or unsaturated (i.e., aromatic) group having from about 3 to 10, preferably about 4 to 8, and more preferably about 5 to 7, carbon atoms, wherein the ring structure includes about one to four heteroatoms. Heterocyclic groups include pyrrolidine, oxolane, thiolane, imidazole, oxazole, piperidine, piperazine, morpholine. The heterocyclic ring can be substituted at one or more positions with such substituents as, for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, other heterocycles, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, $-CF_3$, $-CN$, or the like. Heterocycles may also be bridged or fused to other cyclic groups as described below.

The term "polycyclic group" as used herein is intended to refer to two or more saturated or unsaturated (i.e., aromatic) cyclic rings in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycyclic group can be substituted with such substituents as described above, as for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, $-CF_3$, $-CN$, or the like.

37

A preferred polycyclic group is a group containing a cis-decalin structure. Although
not intending to be limited by mechanism, it is thought that the "bent" conformation
conferred on a modifying group by the presence of a cis-decalin structure contributes to the
efficacy of the modifying group in disrupting Aβ polymerization. Accordingly, other

5   structures which mimic the "bent" configuration of the cis-decalin structure can also be used
as modifying groups. An example of a cis-decalin containing structure that can be used as a
modifying group is a cholanoyl structure, such as a cholyl group. For example, a modulator
compound can be modified at its amino terminus with a cholyl group by reacting the
aggregation core domain with cholic acid, a bile acid, as described in Example 4 (the

10   structure of cholic acid is illustrated in Figure 2). Moreover, a modulator compound can be
modified at its carboxy terminus with a cholyl group according to methods known in the art
(see *e.g.*, Wess, G. *et al.* (1993) *Tetrahedron Letters*, 34:817-822; Wess, G. *et al.* (1992)
Tetrahedron Letters 33:195-198; and Kramer, W. *et al.* (1992) *J. Biol. Chem.* 267:18598-
18604). Cholyl derivatives and analogues can also be used as modifying groups. For

15   example, a preferred cholyl derivative is Aic (3-(*O*-aminoethyl-*iso*)-cholyl), which has a free
amino group that can be used to further modify the modulator compound (*e.g.*, a chelation
group for ^{99m}Tc can be introduced through the free amino group of Aic). As used herein, the
term "cholanoyl structure" is intended to include the cholyl group and derivatives and
analogues thereof, in particular those which retain a four-ring cis-decalin configuration.

20   Examples of cholanoyl structures include groups derived from other bile acids, such as
deoxycholic acid, lithocholic acid, ursodeoxycholic acid, chenodeoxycholic acid and
hyodeoxycholic acid, as well as other related structures such as cholanic acid, bufalin and
resibufogenin (although the latter two compounds are not preferred for use as a modifying
group). Another example of a *cis*-decalin containing compound is 5β-cholestan-3α-ol (the

25   *cis*-decalin isomer of (+)-dihydrocholesterol). For further description of bile acid and steroid
structure and nomenclature, see Nes, W.R. and McKean, M.L. *Biochemistry of Steroids and
Other Isopentanoids*, University Park Press, Baltimore, MD, Chapter 2.

In addition to cis-decalin containing groups, other polycyclic groups may be used as
modifying groups. For example, modifying groups derived from steroids or β-lactams may

30   be suitable modifying groups. Moreover, non-limiting examples of some additional cyclic,
heterocyclic or polycyclic compounds which can be used to modify an Aβ-derived peptidic
structure are shown schematically in Figure 2. In one embodiment, the modifying group is a
"biotinyl structure", which includes biotinyl groups and analogues and derivatives thereof
(such as a 2-iminobiotinyl group). In another embodiment, the modifying group can

35   comprise a "fluorescein-containing group", such as a group derived from reacting an Aβ-
derived peptidic structure with 5-(and 6-)-carboxyfluorescein, succinimidyl ester or
fluorescein isothiocyanate. In various other embodiments, the modifying group(s) can
comprise an *N*-acetylneuraminyl group, a *trans*-4-cotininecarboxyl group, a 2-imino-1-
imidazolidineacetyl group, an (*S*)-(-)-indoline-2-carboxyl group, a (-)-menthoxyacetyl group,

3-3

a 2-norbornaneacetyl group, a γ-oxo-5-acenaphthenebutyryl, a (-)-2-oxo-4-thiazolidinecarboxyl group, a tetrahydro-3-furoyl group, a 2-iminobiotinyl group, a diethylenetriaminepentaacetyl group, a 4-morpholinecarbonyl group, a 2-thiopheneacetyl group or a 2-thiophenesulfonyl group.

5   Preferred modifying groups include groups comprising cholyl structures, biotinyl structures, fluorescein-containing groups, a diethylene-triaminepentaacetyl group, a (-)-menthoxyacetyl group, and a N-acetylneuraminyl group. More preferred modifying groups those comprising a cholyl structure or an iminiobiotinyl group.

In addition to the cyclic, heterocyclic and polycyclic groups discussed above, other

10   types of modifying groups can be used in a modulator of the invention. For example, small hydrophobic groups may be suitable modifying groups. An example of a suitable non-cyclic modifying group is an acetyl group.

Yet another type of modifying group is a compound that contains a non-natural amino acid that acts as a beta-turn mimetic, such as a dibenzofuran-based amino acid described in

15   Tsang. K.Y. et al. (1994) *J. Am. Chem. Soc.* 116:3988-4005; Diaz. H and Kelly. J.W. (1991) *Tetrahedron Letters* 41:5725-5728; and Diaz. H et al. (1992) *J. Am. Chem. Soc.* 114:8316-8318. An example of such a modifying group is a peptide-aminoethyldibenzofuranyl-proprionic acid (Adp) group (e.g., DDIIL-Adp). This type of modifying group further can comprise one or more N-methyl peptide bonds to introduce additional steric hindrance to the

20   aggregation of natural β-AP when compounds of this type interact with natural β-AP.

III.   Additional Chemical Modifications of Aβ Modulators

A β-amyloid modulator compound of the invention can be further modified to alter the specific properties of the compound while retaining the ability of the compound to alter

25   Aβ aggregation and inhibit Aβ neurotoxicity. For example, in one embodiment, the compound is further modified to alter a pharmacokinetic property of the compound, such as *in vivo* stability or half-life. In another embodiment, the compound is further modified to label the compound with a detectable substance. In yet another embodiment, the compound is further modified to couple the compound to an additional therapeutic moiety.

30   Schematically, a modulator of the invention comprising an Aβ aggregation core domain coupled directly or indirectly to at least one modifying group can be illustrated as MG-ACD, whereas this compound which has been further modified to alter the properties of the modulator can be illustrated as MG-ACD-CM, wherein CM represents an additional chemical modification.

35   To further chemically modify the compound, such as to alter the pharmacokinetic properties of the compound. reactive groups can be derivatized. For example, when the modifying group is attached to the amino-terminal end of the aggregation core domain, the carboxy-terminal end of the compound can be further modified. Preferred C-terminal modifications include those which reduce the ability of the compound to act as a substrate for

29

carboxypeptidases. Examples of preferred C-terminal modifiers include an amide group, an ethylamide group and various non-natural amino acids, such as D-amino acids and β-alanine. Alternatively, when the modifying group is attached to the carboxy-terminal end of the aggregation core domain, the amino-terminal end of the compound can be further modified,

5   for example, to reduce the ability of the compound to act as a substrate for aminopeptidases.

A modulator compound can be further modified to label the compound by reacting the compound with a detectable substance. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline

10   phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include

15   ^{14}C, ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{99m}Tc, ^{35}S or ^3H. In a preferred embodiment, a modulator compound is radioactively labeled with ^{14}C, either by incorporation of ^{14}C into the modifying group or one or more amino acid structures in the modulator compound. Labeled modulator compounds can be used to assess the *in vivo* pharmacokinetics of the compounds, as well as to detect Aβ aggregation, for example for diagnostic purposes. Aβ aggregation can

20   be detected using a labeled modulator compound either *in vivo* or in an *in vitro* sample derived from a subject.

Preferably, for use as an *in vivo* diagnostic agent, a modulator compound of the invention is labeled with radioactive technetium or iodine. Accordingly, in one embodiment, the invention provides a modulator compound labeled with technetium, preferably ^{99m}Tc.

25   Methods for labeling peptide compounds with technetium are known in the art (see *e.g.*, U.S. Patent Nos. 5,443,815, 5,225,180 and 5,405,597, all by Dean *et al.*; Stepniak-Biniakiewicz, D., *et al.* (1992) *J. Med. Chem.* 35:274-279; Fritzberg, A.R., *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4025-4029; Baidoo, K.E., *et al.* (1990) *Cancer Res. Suppl.* 50:799s-803s; and Regan, L. and Smith, C.K. (1995) *Science* 270:980-982). A modifying group can be chosen

30   that provides a site at which a chelation group for ^{99m}Tc can be introduced, such as the Aic derivative of cholic acid, which has a free amino group (see Example 11). In another embodiment, the invention provides a modulator compound labeled with radioactive iodine. For example, a phenylalanine residue within the Aβ sequence (such as Phe_{19} or Phe_{20}) can be substituted with radioactive iodotyrosyl (see Example 11). Any of the various isotopes of

35   radioactive iodine can be incorporated to create a diagnostic agent. Preferably, ^{123}I (half-life = 13.2 hours) is used for whole body scintigraphy, ^{124}I (half life = 4 days) is used for positron emission tomography (PET), ^{125}I (half life = 60 days) is used for metabolic turnover studies and ^{131}I (half life = 8 days) is used for whole body counting and delayed low resolution imaging studies.

36

Furthermore, an additional modification of a modulator compound of the invention can serve to confer an additional therapeutic property on the compound. That is, the additional chemical modification can comprise an additional functional moiety. For example, a functional moiety which serves to break down or dissolve amyloid plaques can be coupled

5   to the modulator compound. In this form, the MG-ACD portion of the modulator serves to target the compound to Aβ peptides and disrupt the polymerization of the Aβ peptides, whereas the additional functional moiety serves to break down or dissolve amyloid plaques after the compound has been targeted to these sites.

In an alternative chemical modification, a β-amyloid compound of the invention is

10   prepared in a "prodrug" form, wherein the compound itself does not modulate Aβ aggregation, but rather is capable of being transformed, upon metabolism *in vivo*, into a β-amyloid modulator compound as defined herein. For example, in this type of compound, the modulating group can be present in a prodrug form that is capable of being converted upon metabolism into the form of an active modulating group. Such a prodrug form of a

15   modifying group is referred to herein as a "secondary modifying group." A variety of strategies are known in the art for preparing peptide prodrugs that limit metabolism in order to optimize delivery of the active form of the peptide-based drug (see *e.g.*, Moss, J. (1995) in Peptide-Based Drug Design: Controlling Transport and Metabolism, Taylor, M.D. and Amidon, G.L. (eds), Chapter 18. Additionally strategies have been specifically tailored to

20   achieving CNS delivery based on "sequential metabolism" (see *e.g.*, Bodor, N., *et al.* (1992) *Science* 257:1698-1700; Prokai, L., *et al.* (1994) *J. Am. Chem. Soc.* 116:2643-2644; Bodor, N. and Prokai, L. (1995) in Peptide-Based Drug Design: Controlling Transport and Metabolism, Taylor, M.D. and Amidon, G.L. (eds), Chapter 14. In one embodiment of a prodrug form of a modulator of the invention, the modifying group comprises an alkyl ester

25   to facilitate blood-brain barrier permeability.

Modulator compounds of the invention can be prepared by standard techniques known in the art. The peptide component of a modulator composed, at least in part, of a peptide, can be synthesized using standard techniques such as those described in Bodansky,

30   M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant, G.A (ed.). Synthetic Peptides: A User's Guide, W.H. Freeman and Company, New York (1992). Automated peptide synthesizers are commercially available (*e.g.*, Advanced ChemTech Model 396; Milligen/ Biosearch 9600). Additionally, one or more modulating groups can be attached to the Aβ-derived peptidic component (*e.g.*, an Aβ aggregation core domain) by

35   standard methods, for example using methods for reaction through an amino group (*e.g.*, the alpha-amino group at the amino-terminus of a peptide), a carboxyl group (*e.g.*, at the carboxy terminus of a peptide), a hydroxyl group (*e.g.*, on a tyrosine, serine or threonine residue) or other suitable reactive group on an amino acid side chain (see *e.g.*, Greene, T.W and Wuts, P.G.M. Protective Groups in Organic Synthesis, John Wiley and Sons, Inc., New York

31

(1991). Exemplary syntheses of preferred β amyloid modulators is described further in Examples 1, 4 and 11.

IV. Screening Assays

Another aspect of the invention pertains to a method for selecting a modulator of β-amyloid aggregation. In the method, a test compound is contacted with natural β amyloid peptides, the aggregation of the natural β-AP is measured and a modulator is selected based on the ability of the test compound to alter the aggregation of the natural β-AP (e.g., inhibit or promote aggregation). In a preferred embodiment, the test compound is contacted with a molar excess amount of the natural β-AP. The amount and/or rate of natural β-AP aggregation in the presence of the test compound can be determined by a suitable assay indicative of β-AP aggregation, as described herein (see e.g., Examples 2, 5 and 6).

In a preferred assay, the natural β-AP is dissolved in solution in the presence of the test compound and aggregation of the natural β-AP is assessed in a nucleation assay (see Example 6) by assessing the turbidity of the solution over time, as measured by the apparent absorbance of the solution at 405 nm (described further in Example 6; see also Jarrett et al. (1993) Biochemistry 32:4693-4697). In the absence of a β-amyloid modulator, the A_{405nm} of the solution typically stays relatively constant during a lag time in which the β-AP remains in solution, but then the A_{405nm} of the solution rapidly increases as the β-AP aggregates and comes out of solution, ultimately reaching a plateau level (i.e., the A_{405nm} of the solution exhibits sigmoidal kinetics over time). In contrast, in the presence of a test compound that inhibits β-AP aggregation, the A_{405nm} of the solution is reduced compared to when the modulator is absent. Thus, in the presence of the inhibitory modulator, the solution may exhibit an increased lag time, a decreased slope of aggregation and/or a lower plateau level compared to when the modulator is absent. This method for selecting a modulator of β-amyloid polymerization can similarly be used to select modulators that promote β-AP aggregation. Thus, in the presence of a modulator that promotes β-AP aggregation, the A_{405nm} of the solution is increased compared to when the modulator is absent (e.g., the solution may exhibit an decreased lag time, increase slope of aggregation and/or a higher plateau level compared to when the modulator is absent).

Another assay suitable for use in the screening method of the invention, a seeded extension assay, is also described further in Example 6. In this assay, β-AP monomer and an aggregated β-AP "seed" are combined, in the presence and absence of a test compound, and the amount of β-fibril formation is assayed based on enhanced emission of the dye Thioflavine T when contacted with β-AP fibrils. Moreover, β-AP aggregation can be assessed by electron microscopy (EM) of the β-AP preparation in the presence or absence of the modulator. For example, β amyloid fibril formation, which is detectable by EM, is reduced in the presence of a modulator that inhibits β-AP aggregation (i.e., there is a reduced amount or number of β-fibrils in the presence of the modulator), whereas β fibril formation is

32

increased in the presence of a modulator that promotes β-AP aggregation (*i.e.*, there is an increased amount or number of β-fibrils in the presence of the modulator).

An even more preferred assay for use in the screening method of the invention to select suitable modulators is the neurotoxicity assay described in Examples 3 and 10. 5 Compounds are selected which inhibit the formation of neurotoxic Aβ aggregates and/or which inhibit the neurotoxicity of preformed Aβ fibrils. This neurotoxicity assay is considered to be predictive of neurotoxicity *in vivo*. Accordingly, inhibitory activity of a modulator compound in the *in vitro* neurotoxicity assay is predictive of similar inhibitory activity of the compound for neurotoxicity *in vivo*.

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V. Pharmaceutical Compositions

Another aspect of the invention pertains to pharmaceutical compositions of the β-amyloid modulator compounds of the invention. In one embodiment, the composition includes a β amyloid modulator compound in a therapeutically or prophylactically effective 15 amount sufficient to alter, and preferably inhibit, aggregation of natural β-amyloid peptides, and a pharmaceutically acceptable carrier. In another embodiment, the composition includes a β amyloid modulator compound in a therapeutically or prophylactically effective amount sufficient to inhibit the neurotoxicity of natural β-amyloid peptides, and a pharmaceutically acceptable carrier. A "therapeutically effective amount" refers to an amount effective, at 20 dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reduction or reversal or β-amyloid deposition and/or reduction or reversal of Aβ neurotoxicity. A therapeutically effective amount of modulator may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the modulator to elicit a desired response in the individual. Dosage regimens may be adjusted to 25 provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the modulator are outweighed by the therapeutically beneficial effects. The potential neurotoxicity of the modulators of the invention can be assayed using the cell-based assay described in Examples 3 and 10 and a therapeutically effective modulator can be selected which does not exhibit significant neurotoxicity. In a 30 preferred embodiment, a therapeutically effective amount of a modulator is sufficient to alter, and preferably inhibit, aggregation of a molar excess amount of natural β-amyloid peptides. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting the rate of β-amyloid deposition and/or Aβ neurotoxicity in a subject predisposed 35 to β-amyloid deposition. A prophylactically effective amount can be determined as described above for the therapeutically effective amount. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

33

One factor that may be considered when determining a therapeutically or prophylactically effective amount of a β amyloid modulator is the concentration of natural β-AP in a biological compartment of a subject, such as in the cerebrospinal fluid (CSF) of the subject. The concentration of natural β-AP in the CSF has been estimated at 3 nM

5   (Schwartzman, (1994) *Proc. Natl. Acad. Sci. USA* 91:8368-8372). A non-limiting range for a therapeutically or prophylactically effective amounts of a β amyloid modulator is 0.01 nM-10 μM. It is to be noted that dosage values may vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional

10   judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The amount of active compound in the composition may vary according to factors such as the disease state, age, sex, and weight of the individual, each of which may affect the

15   amount of natural β-AP in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of

20   administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent

25   on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption

30   delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Preferably, the carrier is suitable for administration into the central nervous system (*e.g.*, intraspinally or intracerebrally). Alternatively, the carrier can be suitable for intravenous, intraperitoneal or intramuscular administration. In another embodiment, the carrier is suitable for oral administration.

35   Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is

34

contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, 5   liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the 10   use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the modulators can be administered in a time release formulation, for example in a 15   composition which includes a slow release polymer. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic 20   copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., β-amyloid modulator) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. 25   Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient 30   from a previously sterile-filtered solution thereof.

A modulator compound of the invention can be formulated with one or more additional compounds that enhance the solubility of the modulator compound. Preferred compounds to be added to formulations to enhance the solubility of the modulators are cyclodextrin derivatives, preferably hydroxypropyl-γ-cyclodextrin. Drug delivery vehicles 35   containing a cyclodextrin derivative for delivery of peptides to the central nervous system are described in Bodor, N., et al. (1992) Science 257:1698-1700. For the β-amyloid modulators described herein, inclusion in the formulation of hydroxypropyl-γ-cyclodextrin at a concentration 50-200 mM increases the aqueous solubility of the compounds. In addition to increased solubility, inclusion of a cyclodextrin derivative in the formulation may have other

35

beneficial effects, since β-cyclodextrin itself has been reported to interact with the Aβ peptide
and inhibit fibril formation *in vitro* (Camilleri, P., *et al.* (1994) *FEBS Letters* 341:256-258.
Accordingly, use of a modulator compound of the invention in combination with a
cyclodextrin derivative may result in greater inhibition of Aβ aggregation than use of the
5   modulator alone. Chemical modifications of cyclodextrins are known in the art (Hanessian,
S., *et al.* (1995) J. Org. Chem. 60:4786-4797). In addition to use as an additive in a
pharmaceutical composition containing a modulator of the invention, cyclodextrin derivatives
may also be useful as modifying groups and, accordingly, may also be covalently coupled to
an Aβ peptide compound to form a modulator compound of the invention.

10   In another embodiment, a pharmaceutical composition comprising a modulator of the
invention is formulated such that the modulator is transported across the blood-brain barrier
(BBB). Various strategies known in the art for increasing transport across the BBB can be
adapted to the modulators of the invention to thereby enhance transport of the modulators
across the BBB (for reviews of such strategies, see *e.g.*, Pardridge, W.M. (1994) *Trends in*
15   *Biotechnol.* 12:239-245; Van Bree, J.B. *et al.* (1993) *Pharm. World Sci.* 15:2-9; and
Pardridge, W.M. *et al.* (1992) *Pharmacol. Toxicol.* 71:3-10). In one approach, the modulator
is chemically modified to form a prodrug with enhanced transmembrane transport. Suitable
chemical modifications include covalent linking of a fatty acid to the modulator through an
amide or ester linkage (see *e.g.*, U.S. Patent 4,933,324 and PCT Publication WO 89/07938,
20   both by Shashoua; U.S. Patent 5,284,876 by Hesse *et al.*; Toth, I. *et al.* (1994) *J. Drug*
Target. 2:217-239; and Shashoua, V.E. *et al.* (1984) *J. Med. Chem.* 27:659-664) and
glycating the modulator (see *e.g.*, U.S. Patent 5,260,308 by Poduslo *et al.*). Also, N-
acylamino acid derivatives may be used in a modulator to form a "lipidic" prodrug (see *e.g.*,
5,112,863 by Hashimoto *et al.*).

25   In another approach for enhancing transport across the BBB, a peptidic or
peptidomimetic modulator is conjugated to a second peptide or protein, thereby forming a
chimeric protein, wherein the second peptide or protein undergoes absorptive-mediated or
receptor-mediated transcytosis through the BBB. Accordingly, by coupling the modulator to
this second peptide or protein, the chimeric protein is transported across the BBB. The
30   second peptide or protein can be a ligand for a brain capillary endothelial cell receptor ligand.
For example, a preferred ligand is a monoclonal antibody that specifically binds to the
transferrin receptor on brain capillary endothelial cells (see *e.g.*, U.S. Patents 5,182,107 and
5,154,924 and PCT Publications WO 93/10819 and WO 95/02421, all by Friden *et al.*).
Other suitable peptides or proteins that can mediate transport across the BBB include histones
35   (see *e.g.*, U.S. Patent 4,902,505 by Pardridge and Schimmel) and ligands such as biotin,
folate, niacin, pantothenic acid, riboflavin, thiamin, pryridoxal and ascorbic acid (see *e.g.*,
U.S. Patents 5,416,016 and 5,108,921, both by Heinstein). Additionally, the glucose
transporter GLUT-1 has been reported to transport glycopeptides (L-serinyl-β-D-glucoside
analogues of [Met5]enkephalin) across the BBB (Polt, R. *et al.* (1994) *Proc. Natl. Acad. Sci.*

36

USA 91:7114-1778). Accordingly, a modulator compound can be coupled to such a glycopeptide to target the modulator to the GLUT-1 glucose transporter. For example, a modulator compound which is modified at its amino terminus with the modifying group Aic (3-(*O*-aminoethyl-*iso*)-cholyl, a derivative of cholic acid having a free amino group) can be coupled to a glycopeptide through the amino group of Aic by standard methods. Chimeric proteins can be formed by recombinant DNA methods (*e.g.*, by formation of a chimeric gene encoding a fusion protein) or by chemical crosslinking of the modulator to the second peptide or protein to form a chimeric protein. Numerous chemical crosslinking agents are known in the (*e.g.*, commercially available from Pierce, Rockford IL). A crosslinking agent can be chosen which allows for high yield coupling of the modulator to the second peptide or protein and for subsequent cleavage of the linker to release bioactive modulator. For example, a biotin-avidin-based linker system may be used.

In yet another approach for enhancing transport across the BBB, the modulator is encapsulated in a carrier vector which mediates transport across the BBB. For example, the modulator can be encapsulated in a liposome, such as a positively charged unilamellar liposome (see *e.g.*, PCT Publications WO 88/07851 and WO 88/07852, both by Faden) or in polymeric microspheres (see *e.g.*, U.S. Patent 5,413,797 by Khan *et al.*, U.S. Patent 5,271,961 by Mathiowitz *et al.* and 5,019,400 by Gombotz *et al.*). Moreover, the carrier vector can be modified to target it for transport across the BBB. For example, the carrier vector (*e.g.*, liposome) can be covalently modified with a molecule which is actively transported across the BBB or with a ligand for brain endothelial cell receptors, such as a monoclonal antibody that specifically binds to transferrin receptors (see *e.g.*, PCT Publications WO 91/04014 by Collins *et al.* and WO 94/02178 by Greig *et al.*).

In still another approach to enhancing transport of the modulator across the BBB, the modulator is coadministered with another agent which functions to permeabilize the BBB. Examples of such BBB "permeabilizers" include bradykinin and bradykinin agonists (see *e.g.*, U.S. Patent 5,112,596 by Malfroy-Camine) and peptidic compounds disclosed in U.S. Patent 5,268,164 by Kozarich *et al.*

A modulator compound of the invention can be formulated into a pharmaceutical composition wherein the modulator is the only active compound or, alternatively, the pharmaceutical composition can contain additional active compounds. For example, two or more modulator compounds may be used in combination. Moreover, a modulator compound of the invention can be combined with one or more other agents that have anti-amyloidogenic properties. For example, a modulator compound can be combined with the non-specific cholinesterase inhibitor tacrine (Cognex®, Parke-Davis).

In another embodiment, a pharmaceutical composition of the invention is provided as a packaged formulation. The packaged formulation may include a pharmaceutical composition of the invention in a container and printed instructions for administration of the

37

composition for treating a subject having a disorder associated with β–amyloidosis, e.g. Alzheimer's disease.

VI. Methods of Using Aβ Modulators

5   Another aspect of the invention pertains to methods for altering the aggregation or inhibiting the neurotoxicity of natural β-amyloid peptides. In the methods of the invention, natural β amyloid peptides are contacted with a β amyloid modulator such that the aggregation of the natural β amyloid peptides is altered or the neurotoxicity of the natural β amyloid peptides is inhibited. In a preferred embodiment, the modulator inhibits aggregation

10   of the natural β amyloid peptides. In another embodiment, the modulator promotes aggregation of the natural β amyloid peptides. Preferably, aggregation of a molar excess amount of β-AP, relative to the amount of modulator, is altered upon contact with the modulator.

          In the method of the invention, natural β amyloid peptides can be contacted with a

15   modulator either *in vitro* or *in vivo*. Thus, the term "contacted with" is intended to encompass both incubation of a modulator with a natural β-AP preparation *in vitro* and delivery of the modulator to a site *in vivo* where natural β-AP is present. Since the modulator compound interacts with natural β-AP, the modulator compounds can be used to detect natural β-AP, either *in vitro* or *in vivo*. Accordingly, one use of the modulator compounds of the invention

20   is as diagnostic agents to detect the presence of natural β-AP, either in a biological sample or *in vivo* in a subject. Furthermore, detection of natural β-AP utilizing a modulator compound of the invention further can be used to diagnose amyloidosis in a subject. Additionally, since the modulator compounds of the invention disrupt β-AP aggregation and inhibit β-AP neurotoxicity, the modulator compounds also are useful in the treatment of disorders

25   associated with β-amyloidosis, either prophylactically or therapeutically. Accordingly, another use of the modulator compounds of the invention is as therapeutic agents to alter aggregation and/or neurotoxicity of natural β-AP.

          In one embodiment, a modulator compound of the invention is used *in vitro*, for example to detect and quantitate natural β-AP in sample (*e.g.*, a sample of biological fluid).

30   To aid in detection, the modulator compound can be modified with a detectable substance. The source of natural β-AP used in the method can be, for example, a sample of cerebrospinal fluid (*e.g.*, from an AD patient, an adult susceptible to AD due to family history, or a normal adult). The natural β-AP sample is contacted with a modulator of the invention and aggregation of the β-AP is measured, such as by as assay described in

35   Examples 2, 5 and 6. Preferably, the nucleation assay and/or seeded extension assay described in Example 6 is used. The degree of aggregation of the β-AP sample can then be compared to that of a control sample(s) of a known concentration of β-AP, similarly contacted with the modulator and the results can be used as an indication of whether a subject is susceptible to or has a disorder associated with β-amyloidosis. Moreover, β-AP can be

33

detected by detecting a modulating group incorporated into the modulator. For example, modulators incorporating a biotin compound as described herein (e.g., an amino-terminally biotinylated β-AP peptide) can be detected using a streptavidin or avidin probe which is labeled with a detectable substance (e.g., an enzyme, such as peroxidase). Detection of

5   natural β-AP aggregates mixed with a modulator of the invention using a probe that binds to the modulating group (e.g., biotin/streptavidin) is described further in Example 2.

In another embodiment, a modulator compound of the invention is used *in vivo* to detect, and, if desired, quantitate, natural β-AP deposition in a subject, for example to aid in the diagnosis of β amyloidosis in the subject. To aid in detection, the modulator compound

10   can be modified with a detectable substance, preferably ^{99m}Tc or radioactive iodine (described further above), which can be detected *in vivo* in a subject. The labeled β-amyloid modulator compound is administered to the subject and, after sufficient time to allow accumulation of the modulator at sites of amyloid deposition, the labeled modulator compound is detected by standard imaging techniques. The radioactive signal generated by

15   the labeled compound can be directly detected (e.g., whole body counting), or alternatively, the radioactive signal can be converted into an image on an autoradiograph or on a computer screen to allow for imaging of amyloid deposits in the subject. Methods for imaging amyloidosis using radiolabeled proteins are known in the art. For example, serum amyloid P component (SAP), radiolabeled with either ^{123}I or ^{99m}Tc, has been used to image systemic

20   amyloidosis (see e.g., Hawkins, P.N. and Pepys, M.B. (1995) *Eur. J. Nucl. Med.* 22:595-599). Of the various isotypes of radioactive iodine, preferably ^{123}I (half-life = 13.2 hours) is used for whole body scintigraphy, ^{124}I (half life = 4 days) is used for positron emission tomography (PET), ^{125}I (half life = 60 days) is used for metabolic turnover studies and ^{131}I (half life = 8 days) is used for whole body counting and delayed low resolution imaging

25   studies. Analogous to studies using radiolabeled SAP, a labeled modulator compound of the invention can be delivered to a subject by an appropriate route (e.g., intravenously, intraspinally, intracerebrally) in a single bolus, for example containing 100 μg of labeled compound carrying approximately 180 MBq of radioactivity.

The invention provides a method for detecting the presence or absence of natural β-

30   amyloid peptides in a biological sample, comprising contacting a biological sample with a compound of the invention and detecting the compound bound to natural β-amyloid peptides to thereby detect the presence or absence of natural β-amyloid peptides in the biological sample. In one embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid modulator compound is

35   contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine.

The invention also provides a method for detecting natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease, comprising contacting a biological sample

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with the compound of the invention and detecting the compound bound to natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease. In one embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine. Preferably, use of the method facilitates diagnosis of Alzheimer's disease.

In another embodiment, the invention provides a method for altering natural β-AP aggregation or inhibiting β-AP neurotoxicity, which can be used prophylactically or therapeutically in the treatment or prevention of disorders associated with β amyloidosis, *e.g.*, Alzheimer's Disease. As demonstrated in Example 10, modulator compounds of the invention reduce the toxicity of natural β-AP aggregates to cultured neuronal cells. Moreover, the modulators not only reduce the formation of neurotoxic aggregates but also have the ability to reduce the neurotoxicity of preformed Aβ fibrils. Accordingly, the modulator compounds of the invention can be used to inhibit or prevent the formation of neurotoxic Aβ fibrils in subjects (*e.g.*, prophylactically in a subject predisposed to β-amyloid deposition) and can be used to reverse β-amyloidosis therapeutically in subjects already exhibiting β-amyloid deposition.

A modulator of the invention is contacted with natural β amyloid peptides present in a subject (*e.g.*, in the cerebrospinal fluid or cerebrum of the subject) to thereby alter the aggregation of the natural β-AP and/or inhibit the neurotoxicity of the natural β-APs. A modulator compound alone can be administered to the subject, or alternatively, the modulator compound can be administered in combination with other therapeutically active agents (*e.g.*, as discussed above in subsection IV). When combination therapy is employed, the therapeutic agents can be coadministered in a single pharmaceutical composition, coadministered in separate pharmaceutical compositions or administered sequentially.

The modulator may be administered to a subject by any suitable route effective for inhibiting natural β-AP aggregation in the subject, although in a particularly preferred embodiment, the modulator is administered parenterally, most preferably to the central nervous system of the subject. Possible routes of CNS administration include intraspinal administration and intracerebral administration (*e.g.*, intracerebrovascular administration). Alternatively, the compound can be administered, for example, orally, intraperitoneally, intravenously or intramuscularly. For non-CNS administration routes, the compound can be administered in a formulation which allows for transport across the BBB. Certain modulators may be transported across the BBB without any additional further modification whereas others may need further modification as described above in subsection IV.

Suitable modes and devices for delivery of therapeutic compounds to the CNS of a subject are known in the art, including cerebrovascular reservoirs (*e.g.*, Ommaya or Rikker reservoirs; see *e.g.*, Raney, J.P. *et al.* (1988) *J. Neurosci. Nurs.* <u>20</u>:23-29; Sundaresan, N. *et*

al. (1989) *Oncology* 3:15-22), catheters for intrathecal delivery (*e.g.*, Port-a-Cath, Y-catheters
and the like; see *e.g.*, Plummer, J.L. (1991) *Pain* 44:215-220; Yaksh, T.L. *et al.* (1986)
Pharmacol. Biochem. Behav. 25:483-485), injectable intrathecal reservoirs (*e.g.*, Spinalgesic;
see *e.g.*, Brazenor, G.A. (1987) *Neurosurgery* 21:484-491), implantable infusion pump

5   systems (*e.g.*, Infusaid; see *e.g.*, Zierski, J. *et al.* (1988) *Acta Neurochem. Suppl.* 43:94-99;
Kanoff, R.B. (1994) *J. Am. Osteopath. Assoc.* 94:487-493) and osmotic pumps (sold by Alza
Corporation). A particularly preferred mode of administration is via an implantable,
externally programmable infusion pump. Suitable infusion pump systems and reservoir
systems are also described in U.S. Patent No. 5, 368,562 by Blomquist and U.S. Patent No.

10   4,731,058 by Doan, developed by Pharmacia Deltec Inc.

       The method of the invention for altering β-AP aggregation *in vivo*, and in particular
for inhibiting β-AP aggregation, can be used therapeutically in diseases associated with
abnormal β amyloid aggregation and deposition to thereby slow the rate of β amyloid
deposition and/or lessen the degree of β amyloid deposition, thereby ameliorating the course

15   of the disease. In a preferred embodiment, the method is used to treat Alzheimer's disease
(*e.g.*, sporadic or familial AD, including both individuals exhibiting symptoms of AD and
individuals susceptible to familial AD). The method can also be used prophylactically or
therapeutically to treat other clinical occurrences of β amyloid deposition, such as in Down's
syndrome individuals and in patients with hereditary cerebral hemorrhage with amyloidosis-

20   Dutch-type (HCHWA-D). While inhibition of β-AP aggregation is a preferred therapeutic
method, modulators that promote β-AP aggregation may also be useful therapeutically by
allowing for the sequestration of β-AP at sites that do not lead to neurological impairment.

       Additionally, abnormal accumulation of β-amyloid precursor protein in muscle fibers
has been implicated in the pathology of sporadic inclusion body myositis (IBM) (Askana, V.

25   et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:1314-1319; Askanas, V. et al. (1995) *Current
Opinion in Rheumatology* 7:486-496). Accordingly, the modulators of the invention can be
used prophylactically or therapeutically in the treatment of disorders in which β-AP, or APP,
is abnormally deposited at non-neurological locations, such as treatment of IBM by delivery
of the modulators to muscle fibers.

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VII. Unmodified Aβ Peptides that Inhibit Aggregation of Natural β-AP

       In addition to the β-amyloid modulators described hereinbefore in which an Aβ
peptide is coupled to a modifying group, the invention also provides β-amyloid modulators
comprised of an unmodified Aβ peptide. It has now been discovered that certain portions of

35   natural β-AP can alter aggregation of natural β-APs when contacted with the natural β-APs
(see Example 12). Accordingly, these unmodified Aβ peptides comprise a portion of the
natural β-AP sequence (*i.e.*, a portion of βAP$_{1-39}$, βAP$_{1-40}$, βAP$_{1-42}$ and βAP$_{1-43}$). In
particular these unmodified Aβ peptides have at least one amino acid deletion compared to
βAP$_{1-39}$, the shortest natural β-AP, such that the compound alters aggregation of natural β-

41

amyloid peptides when contacted with the natural β-amyloid peptides. In various embodiments, these unmodified peptide compounds can promote aggregation of natural β-amyloid peptides, or, more preferably, can inhibit aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Even more preferably, the unmodified

5   peptide compound inhibits aggregation of natural β-amyloid peptides when contacted with a molar excess amount of natural β-amyloid peptides (e.g., a 10-fold, 33-fold or 100-fold molar excess amount of natural β-AP).

As discussed above, the unmodified peptide compounds of the invention comprise an amino acid sequence having at least one amino acid deletion compared to the amino acid

10   sequence of βAP$_{1-39}$. Alternatively, the unmodified peptide compound can have at least five, ten, fifteen, twenty, twenty-five, thirty or thirty-five amino acids deleted compared to βAP$_{1-39}$. Still further the unmodified peptide compound can have 1-5, 1-10, 1-15, 1-20, 1-25, 1-30 or 1-35 amino acids deleted compared to βAP$_{1-39}$. The amino acid deletion(s) may occur at the amino-terminus, the carboxy-terminus, an internal site, or a combination thereof,

15   of the β-AP sequence. Accordingly, in one embodiment, an unmodified peptide compound of the invention comprises an amino acid sequence which has at least one internal amino acid deleted compared to βAP$_{1-39}$. Alternatively, the unmodified peptide compound can have at least five, ten, fifteen, twenty, twenty-five, thirty or thirty-five internal amino acids deleted compared to βAP$_{1-39}$. Still further the unmodified peptide compound can have 1-5, 1-10, 1-

20   15, 1-20, 1-25, 1-30 or 1-35 internal amino acids deleted compared to βAP$_{1-39}$. For peptides with internal deletions, preferably the peptide has an amino terminus corresponding to amino acid residue 1 of natural βAP and a carboxy terminus corresponding to residue 40 of natural βAP and has one or more internal β-AP amino acid residues deleted (i.e., a non-contiguous Aβ peptide).

25   In another embodiment, the unmodified peptide compound comprises an amino acid sequence which has at least one N-terminal amino acid deleted compared to βAP$_{1-39}$. Alternatively, the unmodified peptide compound can have at least five, ten, fifteen, twenty, twenty-five, thirty or thirty-five N-terminal amino acids deleted compared to βAP$_{1-39}$. Still further the unmodified peptide compound can have 1-5, 1-10, 1-15, 1-20, 1-25, 1-30 or 1-35

30   N-terminal amino acids deleted compared to βAP$_{1-39}$.

In yet another embodiment, the unmodified peptide compound comprises an amino acid sequence which has at least one C-terminal amino acid deleted compared to βAP$_{1-39}$. Alternatively, the unmodified peptide compound can have at least five, ten, fifteen, twenty, twenty-five, thirty or thirty-five C-terminal amino acids deleted compared to βAP$_{1-39}$. Still

35   further the unmodified peptide compound can have 1-5, 1-10, 1-15, 1-20, 1-25, 1-30 or 1-35 C-terminal amino acids deleted compared to βAP$_{1-39}$.

In addition to deletion of amino acids as compared to βAP$_{1-39}$, the peptide compound can have additional non-β-AP amino acid residues added to it, for example, at the amino terminus, the carboxy-terminus or at an internal site. In one embodiment, the peptide

42

compound has at least one non-β-amyloid peptide-derived amino acid at its N-terminus.
Alternatively, the compound can have, for example, 1-3, 1-5, 1-7, 1-10, 1-15 or 1-20 non-β-
amyloid peptide-derived amino acid at its N-terminus. In another embodiment, the peptide
compound has at least one non-β-amyloid peptide-derived amino acid at its C-terminus.

5   Alternatively, the compound can have, for example, 1-3, 1-5, 1-7, 1-10, 1-15 or 1-20 non-β-
amyloid peptide-derived amino acid at its C-terminus.

         In specific preferred embodiments, an unmodified peptide compound of the invention
comprises $A\beta_{6-20}$ (the amino acid sequence of which is shown in SEQ ID NO: 4), $A\beta_{16-30}$
(the amino acid sequence of which is shown in SEQ ID NO: 14), $A\beta_{1-20, 26-40}$ (the amino

10   acid sequence of which is shown in SEQ ID NO: 15) or EEVVHHHHQQ-βAP_{16-40} (the
amino acid sequence of which is shown in SEQ ID NO: 16). In the nomenclature used
herein, $\beta AP_{1-20, 26-40}$ represents βAP_{1-40} in which the internal amino acid residues 21-25
have been deleted.

         An unmodified peptide compound of the invention can be chemically synthesized

15   using standard techniques such as those described in Bodansky, M. *Principles of Peptide
Synthesis*, Springer Verlag, Berlin (1993) and Grant, G.A (ed.). *Synthetic Peptides: A User's
Guide*, W.H. Freeman and Company, New York (1992). Automated peptide synthesizers are
commercially available (*e.g.*, Advanced ChemTech Model 396; Milligen/ Biosearch 9600).
Alternatively, unmodified peptide compounds can be prepared according to standard

20   recombinant DNA techniques using a nucleic acid molecule encoding the peptide. A
nucleotide sequence encoding the peptide can be determined using the genetic code and an
oligonucleotide molecule having this nucleotide sequence can be synthesized by standard
DNA synthesis methods (*e.g.*, using an automated DNA synthesizer). Alternatively, a DNA
molecule encoding an unmodified peptide compound can be derived from the natural β-

25   amyloid precursor protein gene or cDNA (*e.g.*, using the polymerase chain reaction and/or
restriction enzyme digestion) according to standard molecular biology techniques.

         Accordingly, the invention further provides an isolated nucleic acid molecule
comprising a nucleotide sequence encoding a β-amyloid peptide compound, the β-amyloid
peptide compound comprising an amino acid sequence having at least one amino acid

30   deletion compared to βAP_{1-39} such that the β-amyloid peptide compound alters aggregation
of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. As used
herein, the term "nucleic acid molecule" is intended to include DNA molecules and RNA
molecules and may be single-stranded or double-stranded, but preferably is double-stranded
DNA. The isolated nucleic acid encodes a peptide wherein one or more amino acids are

35   deleted from the N-terminus, C-terminus and/or an internal site of βAP_{1-39}, as discussed
above. In yet other embodiments, the isolated nucleic acid encodes a peptide compound
having one or more amino acids deleted compared to βAP_{1-39} and further having at least one
non-β-AP derived amino acid residue added to it, for example, at the amino terminus, the
carboxy-terminus or at an internal site. In specific preferred embodiments, an isolated

nucleic acid molecule of the invention encodes βAP_{6-20}, βAP_{16-30}, $\beta AP_{1-20, 26-40}$ or EEVVHHHHQQ-βAP_{16-40}.

To facilitate expression of a peptide compound in a host cell by standard recombinant DNA techniques, the isolated nucleic acid encoding the peptide is
5   incorporated into a recombinant expression vector. Accordingly, the invention also provides recombinant expression vectors comprising the nucleic acid molecules of the invention. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional
10 DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a
15 host cell upon introduction into the host cell. and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general. expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present
20 specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors. such as viral vectors, which serve equivalent functions.

In the recombinant expression vectors of the invention. the nucleotide sequence encoding the peptide compound are operatively linked to one or more regulatory sequences.
25 selected on the basis of the host cells to be used for expression. The term "operably linked" is intended to mean that the sequences encoding the peptide compound are linked to the regulatory sequence(s) in a manner that allows for expression of the peptide compound. The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described. for
30 example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185. Academic Press. San Diego, CA (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell. those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences) and those that direct expression in a regulatable manner (e.g., only in the presence of an
35 inducing agent). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of peptide compound desired, *etc*. The expression vectors of the invention can be introduced into host cells thereby to produce peptide compounds encoded by nucleic acids as described herein.

44

The recombinant expression vectors of the invention can be designed for expression of peptide compounds in prokaryotic or eukaryotic cells. For example, peptide compounds can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel,

5   *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector may be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88

10   (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of proteins or peptides in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39). Examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) *Nature*

15   329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

In addition to the regulatory control sequences discussed above, the recombinant

20   expression vector may contain additional nucleotide sequences. For example, the recombinant expression vector may encode a selectable marker gene to identify host cells that have incorporated the vector. Such selectable marker genes are well known in the art. Moreover, the facilitate secretion of the peptide compound from a host cell, in particular mammalian host cells, the recombinant expression vector preferably encodes a signal

25   sequence operatively linked to sequences encoding the amino-terminus of the peptide compound such that upon expression, the peptide compound is synthesized with the signal sequence fused to its amino terminus. This signal sequence directs the peptide compound into the secretory pathway of the cell and is then cleaved, allowing for release of the mature peptide compound (*i.e.*, the peptide compound without the signal sequence) from

30   the host cell. Use of a signal sequence to facilitate secretion of proteins or peptides from mammalian host cells is well known in the art.

A recombinant expression vector comprising a nucleic acid encoding a peptide compound that alters aggregation of natural β-AP can be introduced into a host cell to thereby produce the peptide compound in the host cell. Accordingly, the invention also

35   provides host cells containing the recombinant expression vectors of the invention. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in

fact, be identical to the parent cell, but are still included within the scope of the term as used herein. A host cell may be any prokaryotic or eukaryotic cell. For example, a peptide compound may be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells. Preferably, the peptide compound is expressed in mammalian cells. In a

5   preferred embodiment, the peptide compound is expressed in mammalian cells *in vivo* in a mammalian subject to treat amyloidosis in the subject through gene therapy (discussed further below). Preferably, the β-amyloid peptide compound encoded by the recombinant expression vector is secreted from the host cell upon being expressed in the host cell.

Vector DNA can be introduced into prokaryotic or eukaryotic cells *via* conventional

10 transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection and viral-mediated transfection. Suitable methods for transforming or

15 transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals. Methods for introducing DNA into mammalian cells *in vivo* are also known in the art and can be used to deliver the vector DNA to a subject for gene therapy purposes (discussed further below).

20 For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those that

25 confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker may be introduced into a host cell on the same vector as that encoding the peptide compound or may be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

30 A nucleic acid of the invention can be delivered to cells *in vivo* using methods known in the art, such as direct injection of DNA, receptor-mediated DNA uptake or viral-mediated transfection. Direct injection has been used to introduce naked DNA into cells *in vivo* (see *e.g.*, Acsadi *et al.* (1991) *Nature* 332: 815-818; Wolff *et al.* (1990) *Science* 247:1465-1468). A delivery apparatus (*e.g.*, a "gene gun") for injecting DNA into cells *in vivo* can be used.

35 Such an apparatus is commercially available (*e.g.*, from BioRad). Naked DNA can also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson *et al.* (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the

46

DNA by receptor-mediated endocytosis. Additionally, a DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano *et al.* (1993)

5   *Proc. Natl. Acad. Sci. USA* 90:2122-2126).

       Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in <u>Current Protocols in Molecular Biology</u>, Ausubel, F.M. *et al.* (eds.) Greene

10   Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes,

15   bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis. *et al.* (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury *et al.* (1991)

20   *Science* 254:1802-1805; van Beusechem *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay *et al.* (1992) *Human Gene Therapy* 3:641-647; Dai *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu *et al.* (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

25   Alternatively, the genome of an adenovirus can be manipulated such that it encodes and expresses a peptide compound but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner *et al.* (1988) *BioTechniques* 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et al.* (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other

30   strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.* (1992) cited *supra*), endothelial cells (Lemarchand *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and

35   Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of

47

insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA).

Adeno-associated virus (AAV) can also be used for delivery of DNA for gene therapy purposes. AAV is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka *et al. Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte *et al.* (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski *et al.* (1989) *J. Virol.* 63:3822-3828; and McLaughlin *et al.* (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. An AAV vector such as that described in Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford *et al.* (1988) *Mol. Endocrinol.* 2:32-39; Tratschin *et al.* (1984) *J. Virol.* 51:611-619; and Flotte *et al.* (1993) *J. Biol. Chem.* 268:3781-3790).

The invention provides a method for treating a subject for a disorder associated with β-amyloidosis, comprising administering to the subject a recombinant expression vector encoding a β-amyloid peptide compound, the compound comprising an amino acid sequence having at least one amino acid deletion compared to βAP$_{1-39}$, such that the β-amyloid peptide compound is synthesized in the subject and the subject is treated for a disorder associated with β-amyloidosis. Preferably, the disorder is Alzheimer's disease. In one embodiment the recombinant expression vector directs expression of the peptide compound in neuronal cells. In another embodiment, the recombinant expression vector directs expression of the peptide compound in glial cells. In yet another embodiment, the recombinant expression vector directs expression of the peptide compound in fibroblast cells.

General methods for gene therapy are known in the art. See for example, U.S. Patent No. 5,399,346 by Anderson *et al.* A biocompatible capsule for delivering genetic material is described in PCT Publication WO 95/05452 by Baetge *et al.* Methods for grafting genetically modified cells to treat central nervous system disorders are described in U.S. Patent No. 5,082,670 and in PCT Publications WO 90/06757 and WO 93/10234, all by Gage *et al.* Isolation and/or genetic modification of multipotent neural stem cells or neuro-derived fetal cells are described in PCT Publications WO 94/02593 by Anderson *et al.*, WO 94/16718 by Weiss *et al.*, and WO 94/23754 by Major *et al.* Fibroblasts transduced with genetic material are described in PCT Publication WO 89/02468 by Mulligan *et al.* Adenovirus vectors for transfering genetic material into cells of the central nervous system are described in PCT Publication WO 94/08026 by Kahn *et al.* Herpes simplex virus vectors suitable for treating neural disorders are described in PCT Publications WO 94/04695 by Kaplitt and WO

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90/09441 by-Geller *et al.* Promoter elements of the glial fibrillary acidic protein that can confer astrocyte specific expression on a linked gene or gene fragment, and which thus can be used for expression of Aβ peptides specifically in astrocytes, is described in PCT Publication WO 93/07280 by Brenner *et al.* Furthermore, alternative to expression of an Aβ peptide to

5   modulate amyloidosis, an antisense oligonucleotide that is complementary to a region of the β-amyloid precursor protein mRNA corresponding to the peptides described herein can be expressed in a subject to modulate amyloidosis. General methods for expressing antisense oligonucleotides to modulate nervous system disorders are described in PCT Publication WO 95/09236.

10   Alternative to delivery by gene therapy, a peptide compound of the invention comprising an amino acid sequence having at least one amino acid deletion compared to βAP_{1-39} can be delivered to a subject by directly administering the peptide compound to the subject as described further herein for the modified peptide compounds of the invention. The peptide compound can be formulated into a pharmaceutical composition comprising a

15   therapeutically effective amount of the β-amyloid peptide compound and a pharmaceutically acceptable carrier. The peptide compound can be contacted with natural β-amyloid peptides with a β-amyloid peptide compound such that aggregation of the natural β-amyloid peptides is inhibited. Moreover, the peptide compound can be administered to the subject in a therapeutically effective amount such that the subject is treated for a disorder associated with

20   β-amyloidosis, such as Alzheimer's disease.

VIII. Other Embodiments

        Although the invention has been illustrated hereinbefore with regard to Aβ peptide compounds, the principles described, involving attachment of a modifying group(s) to a

25   peptide compound, are applicable to any amyloidogenic protein or peptide as a means to create a modulator compound that modulates, and preferably inhibits, amyloid aggregation. Accordingly, the invention provides modulator compounds that can be used to treat amyloidosis in a variety of forms and clinical settings.

        Amyloidosis is a general term used to describe pathological conditions characterized

30   by the presence of amyloid. Amyloid is a general term referring to a group of diverse but specific extracellular protein deposits which are seen in a number of different diseases. Though diverse in their occurrence, all amyloid deposits have common morphologic properties, stain with specific dyes (*e.g.*, Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common

35   ultrastructural features and common x-ray diffraction and infrared spectra. Amyloidosis can be classified clinically as primary, secondary, familial and/or isolated. Primary amyloid appears *de novo* without any preceding disorder. Secondary amyloid is that form which appears as a complication of a previously existing disorder. Familial amyloid is a genetically

44

inherited form found in particular geographic populations. Isolated forms of amyloid are those that tend to involve a single organ system.

Different amyloids are characterized by the type of protein(s) or peptide(s) present in the deposit. For example, as described hereinbefore, amyloid deposits associated with

5   Alzheimer's disease comprise the β-amyloid peptide and thus a modulator compound of the invention for detecting and/or treating Alzheimer's disease is designed based on modification of the β-amyloid peptide. The identities of the protein(s) or peptide(s) present in amyloid deposits associated with a number of other amyloidogenic diseases have been elucidated. Accordingly, modulator compounds for use in the detection and/or treatment of these other

10 amyloidogenic diseases can be prepared in a similar fashion to that described herein for β-AP-derived modulators. *In vitro* assay systems can be established using an amyloidogenic protein or peptide which forms fibrils *in vitro*, analogous to the Aβ assays described herein. Modulators can be identified using such assay systems, based on the ability of the modulator to disrupt the β-sheet structure of the fibrils. Initially, an entire amyloidogenic protein can be

15 modified or, more preferably, a peptide fragment thereof that is known to form fibrils *in vitro* can be modified (*e.g.*, analogous to Aβ1-40 described herein). Amino acid deletion and substitution analyses can then be performed on the modified protein or peptide (analogous to the studies described in the Examples) to delineate an aggregation core domain that is sufficient, when modified, to disrupt fibril formation.

20 Non-limiting examples of amyloidogenic proteins or peptides, and their associated amyloidogenic disorders, include:

Transthyretin (TTR) - Amyloids containing transthyretin occur in familial amyloid polyneuropathy (Portuguese, Japanese and Swedish types), familial amyloid cardiomyopathy (Danish type), isolated cardiac amyloid and systemic senile amyloidosis. Peptide fragments

25 of transthyretin have been shown to form amyloid fibrils *in vitro*. For example, TTR 10-20 and TTR 105-115 form amyloid-like fibrils in 20-30% acetonitrile/water at room temperature (Jarvis, J.A., *et al.*(1994) *Int. J. Pept. Protein Res.* 44:388-398). Moreover, familial cardiomyopathy (Danish type) is associated with mutation of Leu at position 111 to Met, and an analogue of TTR 105-115 in which the wildtype Leu at position 111 has been substituted

30 with Met (TTR 105-115Met111) also forms amyloid-like fibrils *in vitro* (see *e.g.*, Hermansen, L.F., *et al.* (1995) *Eur. J. Biochem.* 227:772-779; Jarvis *et al. supra*). Peptide fragments of TTR that form amyloid fibrils *in vitro* are also described in Jarvis, J.A., *et al.* (1993) *Biochem. Biophys. Res. Commun.* 192:991-998 and Gustavsson, A., *et al.* (1991) *Biochem. Biophys. Res. Commun.* 175:1159-1164. A peptide fragment of wildtype or

35 mutated transthyretin that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of familial amyloid polyneuropathy (Portuguese, Japanese and Swedish types), familial amyloid cardiomyopathy (Danish type), isolated cardiac amyloid or systemic senile amyloidosis.

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Prion Protein (PrP) - Amyloids in a number of spongiform encephalopathies,
including scrapie in sheep, bovine spongiform encephalopathy in cows and Creutzfeldt-Jakob
disease (CJ) and Gerstmann-Straussler-Scheinker syndrome (GSS) in humans, contain PrP.
Limited proteolysis of PrPSc (the prion protein associated with scrapie) leads to a 27-30 kDa

5   fragment (PrP27-30) that polymerizes into rod-shaped amyloids (see *e.g.*, Pan, K.M., *et al.*
(1993) *Proc. Natl. Acad. Sci. USA* 90:10962-10966; Gasset, M., *et al.* (1993) *Proc. Natl.
Acad. Sci. USA* 90:1-5). Peptide fragments of PrP from humans and other mammals have
been shown to form amyloid fibrils *in vitro*. For example, polypeptides corresponding to
sequences encoded by normal and mutant alleles of the PRNP gene (encoding the precursor

10   of the prion protein involved in CJ), in the regions of codon 178 and codon 200,
spontaneously form amyloid fibrils *in vitro* (see *e.g.*, Goldfarb, L.G., *et al.* (1993) *Proc. Natl.
Acad. Sci. USA* 90:4451-4454). A peptide encompassing residues 106-126 of human PrP has
been reported to form straight fibrils similar to those extracted from GSS brains, whereas a
peptide encompassing residues 127-147 of human PrP has been reported to form twisted

15   fibrils resembling scrapie-associated fibrils (Tagliavini. F., *et al.* (1993) *Proc. Natl. Acad.
Sci. USA* 90:9678-9682). Peptides of Syrian hamster PrP encompassing residues 109-122,
113-127, 113-120, 178-191 or 202-218 have been reported to form amyloid fibrils, with the
most amyloidogenic peptide being Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala (SEQ ID NO: 17),
which corresponds to residues 113-120 of Syrian hamster PrP but which is also conserved in

20   PrP from other species (Gasset, M., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10940-
10944). A peptide fragment of PrP that forms amyloid fibrils can be modified as described
herein to create a modulator of amyloidosis that can be used in the detection or treatment of
scrapie. bovine spongiform encephalopathy, Creutzfeldt-Jakob disease or Gerstmann-
Straussler-Scheinker syndrome.

25   Islet Amyloid Polypeptide (IAPP. also known as amylin) - Amyloids containing IAPP
occur in adult onset diabetes and insulinoma. IAPP is a 37 amino acid polypeptide formed
from an 89 amino acid precursor protein (see *e.g.*, Betsholtz, C., *et al.* (1989) *Exp. Cell. Res.*
183:484-493; Westermark, P., *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3881-3885). A
peptide corresponding to IAPP residues 20-29 has been reported to form amyloid-like fibrils

30   *in vitro*. with residues 25-29, having the sequence Ala-Ile-Leu-Ser-Ser (SEQ ID NO: 18),
being strongly amyloidogenic (Westermark, P., *et al.* (1990) *Proc. Natl. Acad. Sci. USA*
87:5036-5040; Glenner. G.G., *et al.* (1988) *Biochem. Biophys. Res. Commun.* 155:608-614).
A peptide fragment of IAPP that forms amyloid fibrils can be modified as described herein to
create a modulator of amyloidosis that can be used in the detection or treatment of adult onset

35   diabetes or insulinoma.

            Atrial Natriuretic Factor (ANF) - Amyloids containing ANF are associated with
isolated atrial amyloid (see *e.g.*, Johansson, B., *et al.* (1987) *Biochem. Biophys. Res.
Commun.* 148:1087-1092). ANF corresponds to amino acid residues 99-126 (proANF99-
126) of the ANF prohormone (proANP1-126) (Pucci. A., *et al.* (1991) J. Pathol. 165:235-

241). ANF, or a fragment thereof, that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of isolated atrial amyloid.

Kappa or Lambda Light Chain - Amyloids containing kappa or lambda light chains are associated idiopathic (primary) amyloidosis, myeloma or macroglobulinemia-associated amyloidosis, and primary localized cutaneous nodular amyloidosis associated with Sjogren's syndrome. The structure of amyloidogenic kappa and lambda light chains, including amino acid sequence analysis, has been characterized (see e.g., Buxbaum, J.N., et al. (1990) Ann. Intern. Med. 112:455-464; Schormann, N., et al. (1995) Proc. Natl. Acad. Sci. USA 92:9490-9494; Hurle, M.R., et al. (1994) Proc. Natl. Acad. Sci. USA 91:5446-5450; Liepnieks. J.J., et al. (1990) Mol. Immunol. 27:481-485; Gertz, M.A., et al. (1985) Scand. J. Immunol. 22:245-250; Inazumi, T., et al. (1994) Dermatology 189:125-128). Kappa or lambda light chains, or a peptide fragment thereof that forms amyloid fibrils, can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of idiopathic (primary) amyloidosis, myeloma or macroglobulinemia-associated amyloidosis or primary localized cutaneous nodular amyloidosis associated with Sjogren's syndrome.

Amyloid A - Amyloids containing the amyloid A protein (AA protein), derived from serum amyloid A, are associated with reactive (secondary) amyloidosis (see e.g., Liepnieks, J.J., et al. (1995) Biochim. Biophys. Acta 1270:81-86), familial Mediterranean Fever and familial amyloid nephropathy with urticaria and deafness (Muckle-Wells syndrome) (see e.g., Linke. R.P., et al. (1983) Lab. Invest. 48:698-704). Recombinant human serum amyloid A forms amyloid-like fibrils in vitro (Yamada, T., et al. (1994) Biochim. Biophys. Acta 1226:323-329) and circular dichroism studies revealed a predominant β sheet/turn structure (McCubbin, W.D., et al. (1988) Biochem J. 256:775-783). Serum amyloid A, amyloid A protein or a fragment thereof that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of reactive (secondary) amyloidosis, familial Mediterranean Fever and familial amyloid nephropathy with urticaria and deafness (Muckle-Wells syndrome).

Cystatin C - Amyloids containing a variant of cystatin C are associated with hereditary cerebral hemorrhage with amyloidosis of Icelandic type. The disease is associated with a leucine to glycine mutation at position 68 and cystatin C containing this mutation aggregates in vitro (Abrahamson, M. and Grubb, A. (1994) Proc. Natl. Acad. Sci. USA 91:1416-1420). Cystatin C or a peptide fragment thereof that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of hereditary cerebral hemorrhage with amyloidosis of Icelandic type.

β2 microglobulin - Amyloids containing β2 microglobulin (β2M) are a major complication of long term hemodialysis (see e.g., Stein, G., et al. (1994) Nephrol. Dial. Transplant. 9:48-50; Floege, J., et al. (1992) Kidney Int. Suppl. 38:S78-S85; Maury, C.P. (1990) Rheumatol. Int. 10:1-8). The native β2M protein has been shown to form amyloid

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fibrils *in vitro* (Connors, L.H., *et al.* (1985) Biochem. Biophys. Res. Commun. 131:1063-1068; Ono, K., *et al.* (1994) Nephron 66:404-407). β2M, or a peptide fragment thereof that forms amyloid fibrils, can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of amyloidosis associated with

5   long term hemodialysis.

Apolipoprotein A-I (ApoA-I) - Amyloids containing variant forms of ApoA-I have been found in hereditary non-neuropathic systemic amyloidosis (familial amyloid polyneuropathy III). For example, N-terminal fragments (residues 1-86, 1-92 and 1-93) of an ApoA-I variant having a Trp to Arg mutation at position 50 have been detected in amyloids

10   (Booth, D.R., *et al.* (1995) *QJM* 88:695-702). In another family, a leucine to arginine mutation at position 60 was found (Soutar, A.K., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7389-7393). ApoA-I or a peptide fragment thereof that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of hereditary non-neuropathic systemic amyloidosis.

15   Gelsolin - Amyloids containing variants of gelsolin are associated with familial amyloidosis of Finnish type. Synthetic gelsolin peptides that have sequence homology to wildtype or mutant gelsolins and that form amyloid fibrils *in vitro* are reported in Maury, C.P. *et al.* (1994) *Lab. Invest.* 70:558-564. A nine residue segment surrounding residue 187 (which is mutated in familial gelsolin amyloidosis) was defined as an amyloidogenic region

20   (Maury, *et al., supra*; see also Maury, C.P., *et al.* (1992) *Biochem. Biophys. Res. Commun.* 183:227-231; Maury, C.P. (1991) *J. Clin. Invest.* 87:1195-1199). Gelsolin or a peptide fragment thereof that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of familial amyloidosis of Finnish type.

25   Procalcitonin or calcitonin - Amyloids containing procalcitonin. calcitonin or calcitonin-like immunoreactivity have been detected in amyloid fibrils associated with medullary carcinoma of the thyroid (see *e.g.*, Butler, M. and Khan, S. (1986) Arch. Pathol. Lab. Med. 110:647-649; Sletten, K., *et al.* (1976) J. Exp. Med. 143:993-998). Calcitonin has been shown to form a nonbranching fibrillar structure *in vitro* (Kedar, I., *et al.* (1976) Isr. J.

30   Med. Sci. 12:1137-1140). Procalcitonin. calcitonin or a fragment thereof that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of amyloidosis associated with medullary carcinoma of the thyroid.

Fibrinogen - Amyloids containing a variant form of fibrinogen alpha-chain have been

35   found in hereditary renal amyloidosis. An arginine to leucine mutation at position 554 has been reported in amyloid fibril protein isolated from postmortem kidney of an affected individual (Benson. M.D., *et al.* (1993) *Nature Genetics* 3:252-255). Fibrinogen alpha-chain or a peptide fragment thereof that forms amyloid fibrils can be modified as described herein

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to create a modulator of amyloidosis that can be used in the detection or treatment of fibrinogen-associated hereditary renal amyloidosis.

Lysozyme - Amyloids containing a variant form of lysozyme have been found in hereditary systemic amyloidosis. In one family the disease was associated with a threonine to

5   isoleucine mutation at position 56, whereas in another family the disease was associated with a histidine to aspartic acid mutation at position 67 (Pepys, M.B., *et al.* (1993) *Nature* 362:553-557). Lysozyme or a peptide fragment thereof that forms amyloid fibrils can be modified as described herein to create a modulator of amyloidosis that can be used in the detection or treatment of lysozyme-associated hereditary systemic amyloidosis.

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This invention is further illustrated by the following examples which should not be construed as limiting. A modulator's ability to alter the aggregation of β-amyloid peptide in the assays described below are predictive of the modulator's ability to perform the same function *in vivo*. The contents of all references, patents and published patent applications

15   cited throughout this application are hereby incorporated by reference.

EXAMPLE 1:   Construction of β-Amyloid Modulators

A β-amyloid modulator composed of an amino-terminally biotinylated β-amyloid

20   peptide of the amino acid sequence:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV

(positions 1 to 40 of SEQ ID NO: 1) was prepared by solid-phase peptide synthesis using an N^α-9-fluorenylmethyloxycarbonyl (FMOC)-based protection strategy as follows. Starting with 2.5 mmoles of FMOC-Val-Wang resin. sequential additions of each amino acid were

25   performed using a four-fold excess of protected amino acids. 1-hydroxybenzotriazole (HOBt) and diisopropyl carbodiimide (DIC). Recouplings were performed when necessary as determined by ninhydrin testing of the resin after coupling. Each synthesis cycle was minimally described by a three minute deprotection (25 % piperidine/N-methyl-pyrrolidone (NMP)), a 15 minute deprotection, five one minute NMP washes, a 60 minute coupling cycle.

30   five NMP washes and a ninhydrin test. To a 700 mg portion of the fully assembled peptide-resin. biotin (obtained commercially from Molecular Probes, Inc.) was substituted for an FMOC-amino acid was coupled by the above protocol. The peptide was removed from the resin by treatment with trifluoroacetic acid (TFA) (82.5 %). water (5 %), thioanisole (5 %), phenol (5 %). ethanedithiol (2.5 %) for two hours followed by precipitation of the peptide in

35   cold ether. The solid was pelleted by centrifugation (2400 rpm x 10 min.). and the ether decanted. It was resuspended in ether, pelleted and decanted a second time. The solid was dissolved in 10 % acetic acid and lyophilized to dryness to yield 230 mg of crude biotinylated peptide. 60 mg of the solid was dissolved in 25 % acetonitrile (ACN) /0.1 % TFA and applied to a C18 reversed phase high performance liquid chromatography (HPLC) column.

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Biotinyl βAP$_{1-40}$ was eluted using a linear gradient of 30-45 % acetonitrile/0.1 % TFA over
40 minutes. One primary fraction (4 mg) and several side fractions were isolated. The main
fraction yielded a mass spectrum of 4556 (matrix-assisted laser desorption ionization -time of
flight) which matches the theoretical (4555) for this peptide.

5   A β-amyloid modulator composed of an amino-terminally biotinylated β-amyloid
peptide of the amino acid sequence:
                              DAEFRHDSGYEVHHQ
(positions 1 to 15 of SEQ ID NO: 1) was prepared on an Advanced ChemTech Model 396
multiple peptide synthesizer using an automated protocol established by the manufacturer for

10   0.025 mmole scale synthesis. Double couplings were performed on all cycles using
2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N,N-
diisopropylethylamine (DIEA)/HOBt/FMOC-AA in four-fold excess for 30 minutes followed
by DIC/HOBt/FMOC-AA in four-fold excess for 45 minutes. The peptide was deprotected
and removed from the resin by treatment with TFA/water (95 %/5 %) for three hours and

15   precipitated with ether as described above. The pellet was resuspended in 10 % acetic acid
and lyophilized. The material was purified by a preparative HPLC using 15 %-40 %
acetonitrile over 80 minutes on a Vydac C18 column (21 x 250 mm). The main isolate eluted
as a single symmetrical peak when analyzed by analytical HPLC and yielded the expected
molecular weight when analyzed by electrospray mass spectrometry. Result = 2052.6 (2052

20   theoretical).
          β-amyloid modulator compounds comprising other regions of the β-AP amino acid
sequence (e.g.. an Aβ aggregation core domain) were similarly prepared using the synthesis
methods described above. Moreover, modulators comprising other amyloidogenic peptides
can be similarly prepared.

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EXAMPLE 2:   **Inhibition of β-Amyloid Aggregation by Modulators**

          The ability of β-amyloid modulators to inhibit the aggregation of natural β-AP when
combined with the natural β-AP was examined in a series of aggregation assays. Natural β-

30   AP (β-AP$_{1-40}$) was obtained commercially from Bachem (Torrance, CA). Amino-terminally
biotinylated β-AP modulators were prepared as described in Example 1.

A. Optical Density Assay
          In one assay, β-AP aggregation was measured by determining the increase in turbidity

35   of a solution of natural β-AP over time in the absence or presence of various concentrations
of the modulator. Turbidity of the solution was quantitated by determining the optical
density at 400 nm ($A_{400 nm}$) of the solution over time.
          The aggregation of natural β-AP in the absence of modulator was determined as
follows. β-AP$_{1-40}$ was dissolved in hexafluoro isopropanol (HFIP; Aldrich Chemical Co.,

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Inc.) at 2 mg/ml. Aliquots of the HFIP solution (87 μl) were transferred to individual 10 mm x 75 mm test tubes. A stream of argon gas was passed through each tube to evaporate the HFIP. To the resulting thin film of peptide, dimethylsulfoxide (DMSO; Aldrich Chemical Co., Inc.) (25 μl) was added to dissolve the peptide. A 2 mm x 7 mm teflon-coated magnetic

5   stir bar was added to each tube. Buffer (475 μL of 100 mM NaCl, 10 mM sodium phosphate, pH 7.4) was added to the DMSO solution with stirring. The resulting mixture was stirred continuously and the optical density was monitored at 400 nm to observe the formation of insoluble peptide aggregates.

Alternatively, β-AP$_{1-40}$ was dissolved in DMSO as described above at

10   1.6 mM (6.9 mg/ml) and aliquots (25 μl) were added to stirred buffer (475 μl), followed by monitoring of absorbance at 400 nm.

For inhibition studies in which a β-amyloid modulator was dissolved in solution together with the natural β-AP, the modulators were dissolved in DMSO either with or without prior dissolution in HFIP. These compounds were then added to buffer with stirring,

15   followed by addition of β-AP$_{1-40}$ in DMSO. Alternatively, HFIP solutions of modulators were combined with β-AP$_{1-40}$ in HFIP followed by evaporation and redissolution of the mixture in DMSO. Buffer was then added to the DMSO solution to initiate the assay. The amino-terminally biotinylated β-amyloid peptide modulators N-biotinyl-βAP$_{1-40}$, and N-biotinyl-βAP$_{1-15}$ were tested at concentrations of 1 % and 5 % in the natural β-AP$_{1-40}$

20   solution.

A representative example of the results is shown graphically in Figure 1, which depicts the inhibition of aggregation of natural β-AP$_{1-40}$ by N-biotinyl-βAP$_{1-40}$. In the absence of the modulator, the optical density of the natural β-AP solution showed a characteristic sigmoidal curve, with a lag time prior to aggregation (approximately 3 hours in

25   Figure 1) in which the A$_{400\ nm}$ was low , followed by rapid increase in the A$_{400\ nm}$, which quickly reached a plateau level, representing aggregation of the natural β amyloid peptides. In contrast, in the presence of as little as 1 % of the N-biotinyl-βAP$_{1-40}$ modulator, aggregation of the natural β amyloid peptides was markedly inhibited, indicated by an increase in the lag time, a decrease in the slope of aggregation and a decrease in the plateau

30   level reached for the turbidity of the solution (see Figure 1). N-biotinyl-βAP$_{1-40}$ at a concentration of 5 % similarly inhibited aggregation of the natural β amyloid peptide. Furthermore, similar results were observed when N-biotinyl-βAP$_{1-15}$ was used as the modulator. These results demonstrate that an N-terminally biotinylated β-AP modulator can effectively inhibit the aggregation of natural β amyloid peptides, even when the natural β

35   amyloid peptides are present at as much as a 100-fold molar excess concentration.

B. Fluorescence Assay

In a second assay, β-AP aggregation was measured using a fluorometric assay essentially as described in Levine, H. (1993) *Protein Science* 2:404-410. In this assay, the

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dye thioflavine T (ThT) is contacted with the β-AP solution. Association of ThT with aggregated β-AP, but not monomeric or loosely associated β-AP, gives rise to a new excitation (ex) maximum at 450 nm and an enhanced emission (em) at 482 nm, compared to the 385 nm (ex) and 445 nm (em) for the free dye. β-AP aggregation was assayed by this

5   method as follows. Aliquots (2.9 μl) of the solutions used in the aggregation assays as described above in section A were removed from the samples and diluted in 200 μl of potassium phosphate buffer (50 mM, pH 7.0) containing thioflavin T (10 μM; obtained commercially from Aldrich Chemical Co., Inc.). Excitation was set at 450 nm and emission was measured at 482 nm. Similar to the results observed with the optical density assay

10   described above in section A, as little as 1 % of the N-biotinylated β-AP modulators was effective at inhibiting the aggregation of natural β amyloid peptides using this fluorometric assay.

C. Static Aggregation Assay

15   In a third assay, β-AP aggregation was measured by visualization of the peptide aggregates using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In this assay, β-AP solutions were allowed to aggregate over a period of time and then aliquots of the reaction were run on a standard SDS-PAGE gel. Typical solution conditions were 200 μM of β-AP$_{1-}$
$_{40}$ in PBS at 37 °C for 8 days or 200 μM β-AP$_{1-40}$ in 0.1 M sodium acetate at 37 °C for 3

20   days. The peptide aggregates were visualized by Coomassie blue staining of the gel or, for β-AP solutions that included a biotinylated β-AP modulator, by western blotting of a filter prepared from the gel with a streptavidin-peroxidase probe, followed by a standard peroxidase assay. The β-AP aggregates are identifiable as high molecular weight, low mobility bands on the gel, which are readily distinguishable from the low molecular weight,

25   high mobility β-AP monomer or dimer bands.

When natural β-AP$_{1-40}$ aggregation was assayed by this method in the absence of any β amyloid modulators, high molecular weight aggregates were readily detectable on the gel. In contrast, when N-biotinyl-β-AP$_{1-40}$ modulator self-aggregation was assayed (i.e., aggregation of the N-biotinyl peptide alone, in the absence of any natural β-AP), few if any

30   high molecular weight aggregates were observed, indicating that the ability of the modulator to self-aggregate is significantly reduced compared to natural β-AP. Finally, when aggregation of a mixture of natural β-AP$_{1-40}$ and N-biotinylated β-AP$_{1-40}$ was assayed by this method, reduced amounts of the peptide mixture associated into high molecular weight aggregates, thus demonstrating that the β amyloid modulator is effective at inhibiting the

35   aggregation of the natural β amyloid peptides.

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EXAMPLE 3:   **Neurotoxicity Analysis of β-Amyloid Modulators**

The neurotoxicity of the β-amyloid modulators is tested in a cell-based assay using
the neuronal precursor cell line PC-12, or primary neuronal cells, and the viability indicator
5   3,(4,4-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT). (See Shearman, M.S.
et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:1470-1474; Hansen, M.B. *et al.* (1989) *J. Immun.
Methods* 119:203-210). PC-12 is a rat adrenal pheochromocytoma cell line and is available
from the American Type Culture Collection, Rockville, MD (ATCC CRL 1721). MTT
(commercially available from Sigma Chemical Co.) is a chromogenic substrate that is
10   converted from yellow to blue in viable cells, which can be detected spectrophotometrically.

To test the neurotoxicity of a β-amyloid modulator (either alone or combined with
natural β-AP), cells first are plated in 96-well plates at 7,000-10,000 cells/well and allowed to
adhere by overnight culture at 37 °C. Serial dilutions of freshly dissolved or "aged"
modulators (either alone or combined with natural β-AP) in phosphate buffered saline (PBS)
15   are added to the wells in triplicate and incubation is continued for two or more days. Aged
modulators are prepared by incubating an aqueous solution of the modulator at 37 °C
undisturbed for a prolonged period (*e.g.*, five days or more). For the final two hours of
exposure of the cells to the modulator preparation, MTT is added to the media to a final
concentration of 1 mg/ml and incubation is continued at 37 °C. Following the two hour
20   incubation with MTT, the media is removed and the cells are lysed in isopropanol/0.4N HCl
with agitation. An equal volume of PBS is added to each well and the absorbance of each
well at 570 nm is measured to quantitate viable cells. Alternatively, MTT is solubilized by
addition of 50 % N,N-dimethyl formamide/20 % sodium dodecyl sulfate added directly to the
media in the wells and viable cells are likewise quantitated by measuring absorbance at 570
25   nm. The relative neurotoxicity of a β-amyloid modulator (either alone or in combination
with natural β-AP) is determined by comparison to natural β-AP alone (*e.g.*, β1-40, β1-42),
which exhibits neurotoxicity in this assay and thus can serve as a positive control.

EXAMPLE 4:   **Syntheses of Additional Modified β-Amyloid Peptide Compounds**
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In this example, a series of modified β-APs, having a variety of *N*-terminal or random
side chain modifications were synthesized.

A series of *N*-terminally modified β-amyloid peptides was synthesized using standard
methods. Fully-protected resin-bound peptides corresponding to Aβ(1-15) and Aβ(1-40)
35   were prepared as described in Example 1 on Wang resin to eventually afford carboxyl
terminal peptide acids. Small portions of each peptide resin (13 and 20 μmoles, respectively)
were aliquoted into the wells of the reaction block of an Advanced ChemTech Model 396
Multiple Peptide Synthesizer. The *N*-terminal FMOC protecting group of each sample was
removed in the standard manner with 25% piperidine in NMP followed by extensive washing

58

with NMP.- The unprotected N-terminal α-amino group of each peptide-resin sample was modified using one of the following methods:

Method A, coupling of modifying reagents containing free carboxylic acid groups: The modifying reagent (five equivalents) was predissolved in NMP, DMSO or a mixture of these two solvents. HOBT and DIC (five equivalents of each reagent) were added to the dissolved modifier and the resulting solution was added to one equivalent of free-amino peptide-resin. Coupling was allowed to proceed overnight, followed by washing. If a ninhydrin test on a small sample of peptide-resin showed that coupling was not complete, the coupling was repeated using 1-hydroxy-7-azabenzotriazole (HOAt) in place of HOBt.

Method B, coupling of modifying reagents obtained in preactivated forms: The modifying reagent (five equivalents) was predissolved in NMP, DMSO or a mixture of these two solvents and added to one equivalent of peptide-resin. Diisopropylethylamine (DIEA; six equivalents) was added to the suspension of activated modifier and peptide-resin. Coupling was allowed to proceed overnight, followed by washing. If a ninhydrin test on a small sample of peptide-resin showed that coupling was not complete, the coupling was repeated.

After the second coupling (if required) the N-terminally modified peptide-resins were dried at reduced pressure and cleaved from the resin with removal of side-chain protecting groups as described in Example 1. Analytical reversed-phase HPLC was used to confirm that a major product was present in the resulting crude peptides which were purified using Millipore Sep-Pak cartridges or preparative reverse-phase HPLC. Mass spectrometry was used to confirm the presence of the desired compound in the product.

Method A was used to couple N-acetylneuraminic acid, cholic acid, trans-4-cotininecarboxylic acid, 2-imino-1-imidazolidineacetic acid, (S)-(-)-indoline-2-carboxylic acid, (-)-menthoxyacetic acid, 2-norbornaneacetic acid, γ-oxo-5-acenaphthenebutyric acid, (-)-2-oxo-4-thiazolidinecarboxylic acid, and tetrahydro-3-furoic acid. Method B was used to couple 2-iminobiotin-N-hydroxysuccinimide ester, diethylenetriaminepentaacetic dianhydride, 4-morpholinecarbonyl chloride, 2-thiopheneacetyl chloride, and 2-thiophenesulfonyl chloride.

In a manner similar to the construction of N-terminally modified Aβ(1-15) and Aβ(1-40) peptides described above, N-fluoresceinyl Aβ(1-15) and Aβ(1-40) were prepared in two alternative manners using the preactivated reagents 5-(and 6)-carboxyfluorescein succinimidyl ester and fluorescein-5-isothiocyanate (FITC Isomer I). Both reagents were obtained from Molecular Probes Inc. Couplings were performed using four equivalents of reagent per equivalent of peptide-resin with DIEA added to make the reaction solution basic to wet pH paper. Couplings of each reagent to Aβ(1-15)-resin appeared to be complete after a single overnight coupling. Coupling to Aβ(1-40)-resin was slower as indicated by a positive ninhydrin test and both reagents were recoupled to this peptide-resin overnight in

457

tetrahydrofuran-NMP (1:2 v/v). The resulting *N*-terminally modified peptide-resins were cleaved, deprotected and purified as described in Example A.

In addition to the *N*-fluoresceinyl Aβ peptides described above, a β-amyloid modulator comprised of random modification of Aβ(1-40) with fluorescein was prepared.
5   Aβ(1-40) purchased from Bachem was dissolved in DMSO at approximately 2 mg/mL. 5-(and-6)-Carboxyfluorescein purchased from Molecular Probes was added in a 1.5 molar excess and DIEA was added to make the solution basic to wet pH paper. The reaction was allowed to proceed for 1 hour at room temperature and was then quenched with triethanolamine. The product was added to assays as this crude mixture.
10   β-amyloid modulator compounds comprising other regions of the β-AP amino acid sequence (*e.g.*, an Aβ aggregation core domain) were similarly prepared using the synthesis methods described above. Moreover, modulators comprising other amyloidogenic peptides can be similarly prepared.

15   **EXAMPLE 5:**   **Identification of Additional β-Amyloid Modulators**

In this Example, two assays of Aβ aggregation were used to identify β-amyloid modulators which can inhibit this process.

The first assay is referred to as a seeded static assay (SSA) and was performed as
20   follows:

To prepare a solution of Aβ monomer, the appropriate quantity of Aβ(1-40) peptide (Bachem) was weighed out on a micro-balance (the amount was corrected for the amount of water in the preparation, which, depending on lot number, was 20-30% w/w). The peptide was dissolved in 1/25 volume of dimethysulfoxide (DMSO), followed by water to 1/2
25   volume and 1/2 volume 2x PBS (10x PBS: NaCl 137 mM, KCl 2.7 mM Na₂HPO₄ • 7H₂O 4.3 mM, KH₂PO₄ 1.4 mM pH 7.2) to a final concentration of 200 μM.

To prepare a stock seed, 1 ml of the above Aβ monomer preparation, was incubated for 8 days at 37 °C and sheared sequentially through an 18, 23, 26 and 30 gauge needle 25, 25, 50, and 100 times respectively. 2 μl samples of the sheared material was taken for
30   fluorescence measurements after every 50 passes through the 30 gauge needle until the fluorescence units (FU) had plateaued (approx. 100-150x).

To prepare a candidate inhibitor, the required amount of candidate inhibitor was weighed out and the stock dissolved in 1x PBS to a final concentration of 1 mM (10x stock). If insoluble, it was dissolved in 1/10 volume of DMSO and diluted in 1x PBS to 1 mM. A
35   further 1/10 dilution was also prepared to test each candidate at both 100 μM and 10 μM.

For the aggregation assay, each sample was set up in triplicate [50 μl of 200 μM monomer, 125 FU sheared seed (variable quantity dependent on the batch of seed, routinely 3-6 μl), 10 μl of 10x inhibitor solution, final volume made up to 100 μl with 1x PBS]. Two concentrations of each inhibitor were tested 100 μM and 10 μM, equivalent to a 1:1 and a

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1:10 molar ratio of monomer to inhibitor. The controls included an unseeded reaction to confirm that the fresh monomer contained no seed, and a seeded reaction in the absence of inhibitor, as a reference to compare against putative inhibitors. The assay was incubated at 37 °C for 6 h, taking 2 µl samples hourly for fluorescence measurements. To measure

5   fluorescence, a 2 µl sample of Aβ was added to 400 µl of Thioflavin-T solution (50 mM Potassium Phosphate 10 mM Thioflavin-T pH 7.5). The samples were vortexed and the fluorescence was read in a 0.5 ml micro quartz cuvette at EX 450 nm and EM 482 nm (Hitachi 4500 Fluorimeter). β-aggregation results in enhanced emission of Thioflavin-T. Accordingly, samples including an effective inhibitor compound exhibit reduced emission as

10   compared to control samples without the inhibitor compound.

The second assay is referred to as a shaken plate aggregation assay and was performed as follows:

Aβ(1-40) peptide from Bachem (Torrance, CA) was dissolved in HFIP (1,1,1,3,3,3-

15   Hexafluoro-2-propanol; Aldrich 10.522-8) at a concentration of 2 mg peptide/ml and incubated at room temperature for 30 min. HFIP solubilized peptide was sonicated in a waterbath sonicator for 5 min at highest setting, then evaporated to dryness under a stream of argon. The peptide film was resuspended in anhydrous dimethylsulfoxide (DMSO) at a concentration of 6.9 mg/ml, sonicated for 5 min as before, then filtered through a 0.2 micron nylon syringe filter

20   (VWR cat. No. 28196-050). Candidate inhibitors were dissolved directly in DMSO, generally at a molar concentration 4 times that of the Aβ(1-40) peptide.

Candidates were assayed in triplicate. For each candidate to be tested, 4 parts Aβ(1-40) peptide in DMSO were combined with 1 part candidate inhibitor in DMSO in a glass vial, and mixed to produce a 1:1 molar ratio of Aβ peptide to candidate. For different molar ratios,

25   candidates were diluted with DMSO prior to addition to Aβ(1-40), in order to keep the final DMSO and Aβ(1-40) concentrations constant. Into an ultra low binding 96 well plate (Corning Costar cat. No. 2500, Cambridge MA) 100 µl PTL buffer (150 mM NaCl, 10 mM NaH$_2$PO$_4$; pH 7.4) was aliquotted per well. For each candidate, 10 µl of peptide mixture in DMSO was aliquotted into each of three wells containing buffer. The covered plate was vigorously

30   vortexed on a plate shaker at high speed for 30 seconds. An additional 100 µl of PTL buffer was added to each well and again the plate was vortexed vigorously for 30 sec. Absorbance at 405 nm was immediately read in a plate reader for a baseline reading. The plate was returned to the plate shaker and vortexed at moderate speed for 5 hours at room temperature, with absorbance readings taken at 15-20 min intervals. Increased absorbance indicated aggregation.

35   Accordingly, effective inhibitor compounds cause a decrease in absorbance in the test sample as compared to a control sample without the inhibitor compound.

Representative results of the static seeded assay and shaken plate assay with preferred β-amyloid modulators are shown below in Table I.

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TABLE I

Candidate Inhibitor	Aβ Amino Acids	Modifying Reagent	Effect in shaken plate assay	Effect in Seeded Static Assay *
174	Aβ1-15	Cholic acid	Complete inhibition at 100% conc	++
176	Aβ1-15	Diethylene-triamine penta acetic acid	Decreased Plateau	++
180	Aβ1-15	(-)-Menthoxy acetic acid	None	++
190	Aβ1-15	Fluorescein carboxylic acid (FICO)	Decreased Plateau	++
220	Aβ16-40 mutant	h-EVHHHHQQK-[Aβ (16-40)]-OH	Complete inhibition at 100%, increased lag at 10 %	++
224	Aβ1-40 mutant	$F_{19}F_{20}$->$T_{19}T_{20}$	Increased lag	++
233	A6β-20	Acetic acid	accelerated aggregation at 10% conc	++

*   ++   = A strong inhibitor of aggregation. The rate of aggregation in the presence of the inhibitor was decreased compared to the control by at least 30-50%

These results indicate that β-APs modified by a wide variety of N-terminal modifying groups are effective at modulating β-amyloid aggregation.

EXAMPLE 6:   **Additional β-Amyloid Aggregation Assays**

Most preferably, the ability of β-amyloid modulator compounds to modulate (e.g., inhibit or promote) the aggregation of natural β-AP when combined with the natural β-AP is examined in one or both of the aggregation assays described below. Natural β-AP ($β-AP_{1-40}$) for use in the aggregation assays is commercially available from Bachem (Torrance, CA).

A. Nucleation Assay

The nucleation assay is employed to determine the ability of test compounds to alter (e.g. inhibit) the early events in formation of β-AP fibers from monomeric β-AP. Characteristic of a nucleated polymerization mechanism, a lag time is observed prior to

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nucleation, after which the peptide rapidly forms fibers as reflected in a linear rise in turbidity. The time delay before polymerization of β-AP monomer can be quantified as well as the extent of formation of insoluble fiber by light scattering (turbidity). Polymerization reaches equilibrium when the maximum turbidity reaches a plateau. The turbidity of a

5   solution of natural β-AP in the absence or presence of various concentrations of a β-amyloid modulator compound is determined by measuring the apparent absorbance of the solution at 405nm ($A_{405\ nm}$) over time. The threshold of sensitivity for the measurement of turbidity is in the range of 15-20 µM β-AP. A decrease in turbidity over time in the presence of the modulator. as compared to the turbidity in the absence of the modulator, indicates that the

10   modulator inhibits formation of β-AP fibers from monomeric β-AP. This assay can be performed using stirring or shaking to accelerate polymerization. thereby increasing the speed of the assay. Moreover the assay can be adapted to a 96-well plate format to screen multiple compounds.

To perform the nucleation assay, first $A\beta_{1-40}$ peptide is dissolved in HFIP

15   (1,1,1,3,3,3-Hexafluoro-2-propanol: Aldrich 10.522-8) at a concentration of 2 mg peptide/ml and incubated at room temperature for 30 min. HFIP-solubilized peptide is sonicated in a waterbath sonicator for 5 min at highest setting, then evaporated to dryness under a stream of argon. The peptide film is resuspended in anhydrous dimethylsulfoxide (DMSO) at a concentration of 6.9 mg/ml (25x concentration). sonicated for 5 min as before. then filtered

20   through a 0.2 micron nylon syringe filter (VWR cat. No. 28196-050). Test compounds are dissolved in DMSO at a 100x concentration. Four volumes of 25x $A\beta_{1-40}$ peptide in DMSO are combined with one volume of test compound in DMSO in a glass vial. and mixed to produce a 1:1 molar ratio of Aβ peptide to test compound. For different molar ratios, test compounds are diluted with DMSO prior to addition to $A\beta_{1-40}$, in order to keep the final

25   DMSO and $A\beta_{1-40}$ concentrations constant. Control samples do not contain the test compound. Ten microliters of the mixture is then added to the bottom of a well of a Corning Costar ultra low binding 96-well plate (Corning Costar, Cambridge MA; cat. No. 2500). Ninety microliters of water is added to the well. the plate is shaken on a rotary shaken at a constant speed at room temperature for 30 seconds, an additional 100 µl of 2x PTL buffer (20

30   mM NaH_2PO_4. 300 mM NaCl, pH 7.4) is added to the well. the plate is reshaken for 30 seconds and a baseline (t=0) turbidity reading is taken by measuring the apparent absorbance at 405 nm using a Bio-Rad Model 450 Microplate Reader. The plate is then returned to the shaker and shaken continuously for 5 hours. Turbidity readings are taken at 15 minute intervals.

35   β-amyloid aggregation in the absence of any modulators results in enhanced turbidity of the natural β-AP solution (*i.e.*, an increase in the apparent absorbance at 405 nm over time). Accordingly, a solution including an effective inhibitory modulator compound exhibits reduced turbidity as compared to the control sample without the modulator

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compound (*i.e.*, less apparent absorbance at 405 nm over time as compared to the control sample).

B. Seeded Extension Assay

The seeded extension assay can be employed to measure the rate of Aβ fiber formed in a solution of Aβ monomer following addition of polymeric Aβ fiber "seed". The ability of test compounds to prevent further deposition of monomeric Aβ to previously deposited amyloid is determined using a direct indicator of β-sheet formation using fluorescence. In contrast with the nucleation assay, the addition of seed provides immediate nucleation and continued growth of preformed fibrils without the need for continuous mixing, and thus results in the absence of a lag time before polymerization starts. Since this assay uses static polymerization conditions, the activity of positive compounds in the nucleation assay can be confirmed in this second assay under different conditions and with an additional probe of amyloid structure.

In the seeded extension assay, monomeric $A\beta_{1-40}$ is incubated in the presence of a "seed" nucleus (approximately ten mole percent of Aβ that has been previously allowed to polymerize under controlled static conditions). Samples of the solution are then diluted in thioflavin T (Th-T). The polymer-specific association of Th-T with Aβ produces a fluorescent complex that allows the measurement of the extent of fibril formation (Levine, H. (1993) *Protein Science* 2:404-410). In particular, association of Th-T with aggregated β-AP, but not monomeric or loosely associated β-AP, gives rise to a new excitation (ex) maximum at 450 nm and an enhanced emission (em) at 482 nm, compared to the 385 nm (ex) and 445 nm (em) for the free dye. Small aliquots of the polymerization mixture contain sufficient fibril to be mixed with Th-T to allow the monitoring of the reaction mixture by repeated sampling. A linear growth curve is observed in the presence of excess monomer. The formation of thioflavin T responsive β-sheet fibrils parallels the increase in turbidity observed using the nucleation assay.

A solution of Aβ monomer for use in the seeded extension assay is prepared by dissolving an appropriate quantity of $A\beta_{1-40}$ peptide in 1/25 volume of dimethysulfoxide (DMSO), followed by water to 1/2 volume and 1/2 volume 2x PBS (10x PBS: NaCl 137 mM, KCl 2.7 mM $Na_2HPO_4 \cdot 7H_2O$ 4.3 mM, KH_2PO_4 1.4 mM pH 7.2) to a final concentration of 200 μM. To prepare the stock seed, 1 ml of the Aβ monomer preparation, is incubated for approximately 8 days at 37 °C and sheared sequentially through an 18, 23, 26 and 30 gauge needle 25, 25, 50, and 100 times respectively. 2 μl samples of the sheared material is taken for fluorescence measurements after every 50 passes through the 30 gauge needle until the fluorescence units (FU) plateau (approx. 100-150x). Test compounds are prepared by dissolving an appropriate amount of test compound in 1x PBS to a final concentration of 1 mM (10x stock). If insoluble, the compound is dissolved in 1/10 volume of DMSO and

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diluted in 1x PBS to 1 mM. A further 1/10 dilution is also prepared to test each candidate at both 100 μM and 10 μM.

To perform the seeded extension assay, each sample is set up with 50 μl of 200 μM monomer, 125 FU sheared seed (a variable quantity dependent on the batch of seed, routinely

5   3-6 μl) and 10 μl of 10x modulator solution. The sample volume is then adjusted to a final volume of 100 μl with 1x PBS. Two concentrations of each modulator typically are tested: 100 μM and 10 μM, equivalent to a 1:1 and a 1:10 molar ratio of monomer to modulator. The controls include an unseeded reaction to confirm that the fresh monomer contains no seed, and a seeded reaction in the absence of any modulators, as a reference to compare

10   against candidate modulators. The assay is incubated at 37 °C for 6 h. taking 2 μl samples hourly for fluorescence measurements. To measure fluorescence, a 2 μl sample of Aβ is added to 400 μl of Thioflavin-T solution (50 mM Potassium Phosphate 10 mM Thioflavin-T pH 7.5). The samples are vortexed and the fluorescence is read in a 0.5 ml micro quartz cuvette at EX 450 nm and EM 482 nm (Hitachi 4500 Fluorimeter).

15   β-amyloid aggregation results in enhanced emission of Thioflavin-T. Accordingly, samples including an effective inhibitory modulator compound exhibit reduced emission as compared to control samples without the modulator compound.

EXAMPLE 7:   Effect of Different Amino Acid Subregions of Aβ Peptide on the

20   **Inhibitory Activity of β-Amyloid Modulator Compounds**

To determine the effect of various subregions of $Aβ_{1-40}$ on the inhibitory activity of a a β-amyloid modulator, overlapping Aβ peptide 15mers were constructed. For each 15mer, four different amino-terminal modifiers were tested: a cholyl group, an iminobiotinyl group,

25   an N-acetyl neuraminyl group (NANA) and a 5-(and 6-)-carboxyfluoresceinyl group (FICO). The modulators were evaluated in the nucleation and seeded extension assays described in Example 6.

The results of the nucleation assays are summarized below in Table II. The concentration of $Aβ_{1-40}$ used in the assays was 50 μM. The "mole %" value listed in Table II

30   refers to the % concentration of the test compound relative to $Aβ_{1-40}$. Accordingly, 100% indicates that $Aβ_{1-40}$ and the test compound were equimolar. Mole % values less than 100% indicate that $Aβ_{1-40}$ was in molar excess relative to the test compound (*e.g.*, 10% indicates that $Aβ_{1-40}$ was in 10-fold molar excess relative to the test compound). The results of the nucleation assays for each test compound are presented in Table II in two ways. The "fold

35   increase in lag time". which is a measure of the ability of the compound to delay the onset of aggregation, refers to the ratio of the observed lag time in the presence of the test compound to the observed lag time in the control without the test compound. Accordingly a fold increase in lag time of 1.0 indicates no change in lag time. whereas numbers > 1.0 indicate an increase in lag time. The "% inhibition of plateau". which is a measure of the ability of the

compound to decrease the total amount of aggregation, refers to the reduction of the final turbidity in the presence of the test compound expressed as a percent of the control without the test compound. Accordingly, an inhibitor that abolishes aggregation during the course of the assay will have a % inhibition of 100. N-terminally modified Aβ subregions which exhibited inhibitory activity are indicated in bold in Table II.

Table II

Reference #	N-terminal Modification	Aβ Peptide	Mole %	Fold Increase in Lag Time	% Inhibition of Plateau
PPI-174	**cholyl**	**Aβ$_{1-15}$**	**100**	**>4.5**	**100**
PPI-264	**cholyl**	**Aβ$_{6-20}$**	**100**	**>4.5**	**100**
PPI-269	cholyl	Aβ$_{11-25}$	100	1.5	~0
PPI-274	**cholyl**	**Aβ$_{16-30}$**	**100**	**>4.5**	**100**
PPI-279	**cholyl**	**Aβ$_{21-35}$**	**100**	**1.6**	**51**
PPI-284	**cholyl**	**Aβ$_{26-40}$**	**100**	**>4.5**	**87**
PPI-173	NANA	Aβ$_{1-15}$	100	~1	~0
PPI-266	**NANA**	**Aβ$_{6-20}$**	**100**	**1.3**	**64**
PPI-271	**NANA**	**Aβ$_{11-25}$**	**100**	**1.3**	**77**
PPI-276	NANA	Aβ$_{16-30}$	100	~1	~0
PPI-281	NANA	Aβ$_{21-35}$	100	~1	53
PPI-286	NANA	Aβ$_{26-40}$	100	1.3	~0
PPI-172	Iminobiotinyl	Aβ$_{1-15}$	100	1.2	~0
PPI-267	**Iminobiotinyl**	**Aβ$_{6-20}$**	**100**	**1.6**	**44**
PPI-272	**Iminobiotinyl**	**Aβ$_{11-25}$**	**100**	**1.2**	**40**
PPI-277	**Iminobiotinyl**	**Aβ$_{16-30}$**	**100**	**1.2**	**55**
PPI-282	Iminobiotinyl	Aβ$_{21-35}$	100	~1	66
PPI-287	Iminobiotinyl	Aβ$_{26-40}$	100	2.3	~0
PPI-190	FICO	Aβ$_{1-15}$	100	~1	30
PPI-268	FICO	Aβ$_{6-20}$	100	1.9	~0
PPI-273	**FICO**	**Aβ$_{11-25}$**	**100**	**1.7**	**34**
PPI-278	**FICO**	**Aβ$_{16-30}$**	**100**	**1.6**	**59**
PPI-283	**FICO**	**Aβ$_{21-35}$**	**100**	**1.2**	**25**
PPI-288	**FICO**	**Aβ$_{26-40}$**	**100**	**2**	**75**

These results indicate that certain subregions of Aβ$_{1-40}$, when modified with an appropriate modifying group, are effective at inhibiting the aggregation of Aβ$_{1-40}$. A cholyl group was an effective modifying group for several subregions. Cholic acid alone was tested for inhibitory activity but had no effect on Aβ aggregation. The Aβ$_{6-20}$ subregion exhibited high levels of inhibitory activity when modified with several different modifying groups (cholyl, NANA, iminobiotinyl), with cholyl-Aβ$_{6-20}$ (PPI-264) being the most active form.

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Accordingly, this modulator compound was chosen for further analysis, described in Example 8.

EXAMPLE 8: Identification of a Five Amino Acid Subregion of Aβ Peptide
Sufficient for Inhibitory Activity of a β-Amyloid Modulator Compound

To further delineate a minimal subregion of cholyl-$A\beta_{6-20}$ sufficient for inhibitory activity, a series of amino terminal and carboxy terminal amino acid deletions of cholyl-$A\beta_{6-20}$ were constructed. The modulators all had the same cholyl amino-terminal modification. Additionally, for the peptide series having carboxy terminal deletions, the carboxy terminus was further modified to an amide. The modulators were evaluated as described in Example 7 and the results are summarized below in Table III, wherein the data is presented as described in Example 7.

Table III

Ref. #	N-Term. Mod.	Aβ Peptide	C-Term. Mod.	Mole %	Fold Increase in Lag Time	% Inhibition of Plateau
PPI-264	**cholyl**	**$A\beta_{6-20}$**	**-**	**100**	**>4.5**	**100**
				10	2	43
PPI-341	**cholyl**	**$A\beta_{7-20}$**	**-**	**100**	**>4.5**	**100**
				33	2	~0
PPI-342	cholyl	$A\beta_{8-20}$	-	100	1.5	~0
				33	2.1	~0
PPI-343	cholyl	$A\beta_{9-20}$	-	33	2.0	~0
PPI-344	cholyl	$A\beta_{10-20}$	-	33	2.1	~0
PPI-345	cholyl	$A\beta_{11-20}$	-	33	1.5	~0
PPI-346	cholyl	$A\beta_{12-20}$	-	33	2.1	~0
PPI-347	cholyl	$A\beta_{13-20}$	-	33	2.6	~0
PPI-348	**cholyl**	**$A\beta_{14-20}$**	**-**	**33**	**2.0**	**49**
PPI-349	**cholyl**	**$A\beta_{15-20}$**	**-**	**33**	**2.3**	**50**
PPI-350	**cholyl**	**$A\beta_{16-20}$**	**-**	**38**	**3.4**	**23**
PPI-296	cholyl	$A\beta_{6-20}$	amide	33	1.8	~0
PPI-321	cholyl	$A\beta_{6-19}$	amide	33	1.4	~0
PPI-325	cholyl	$A\beta_{6-17}$	amide	33	1.8	~0
PPI-331	cholyl	$A\beta_{6-14}$	amide	33	1.0	29
PPI-339	cholyl	$A\beta_{6-10}$	amide	33	1.1	13

These results indicate that activity of the modulator is maintained when amino acid residue 6 is removed from the amino terminal end of the modulator (*i.e.*, cholyl-$A\beta_{7-20}$ retained activity) but activity is lost as the peptide is deleted further at the amino-terminal end by removal of amino acid position 7 through to amino acid position 12 (*i.e.*, cholyl-$A\beta_{8-20}$

through cholyl-Aβ_{13-20} did inhibit the plateau level of Aβ aggregation). However, further deletion of amino acid position 13 resulted in a compound (i.e., cholyl-Aβ_{14-20}) in which inhibitory activity is restored. Furthermore, additional deletion of amino acid position 14 (i.e., cholyl-Aβ_{15-20}) or positions 14 and 15 (i.e., cholyl-Aβ_{16-20}) still maintained inhibitory activity. Thus, amino terminal deletions of Aβ_{6-20} identified Aβ_{16-20} as a minimal subregion which is sufficient for inhibitory activity when appropriately modified. In contrast, carboxy terminal deletion of amino acid position 20 resulted in loss of activity which was not fully restored as the peptide was deleted further at the carboxy-terminal end. Thus, maintenance of position 20 within the modulator may be important for inhibitory activity.

EXAMPLE 9:   **Identification of a Four Amino Acid Subregion of Aβ Peptide Sufficient for Inhibitory Activity of a β-Amyloid Modulator Compound**

In this example, the smallest effective modulator identified in the studies described in Example 8. cholyl-Aβ_{16-20} (PPI-350), was analyzed further. Additional amino- and carboxy-terminal deletions were made with cholyl-Aβ_{16-20}, as well as an amino acid substitution (Val$_{18}$->Thr), to identify the smallest region sufficient for the inhibitory activity of the modulator. A peptide comprised of five alanine residues, (Ala)$_5$, modified at its amino-terminus with cholic acid, was used as a specificity control. The modulators were evaluated as described in Example 7 and the results are summarized below in Table IV, wherein the data is presented as described in Example 7.

Table IV

Ref. #	N-Term. Mod.	Aβ Peptide	C-Term. Mod.	Mole %	Fold Increase in Lag Time	% Inhibition of Plateau
PPI-264	cholyl	Aβ_{6-20}	-	10	2.0	43
PPI-347	cholyl	Aβ_{13-20}	-	10	2.2	57
PPI-349	cholyl	Aβ_{15-20}	-	100	>5.0	100
				33	2.6	35
				10	2.1	~0
PPI-350	cholyl	Aβ_{16-20}	-	100	>5.0	100
				10	2.4	40
PPI-368	cholyl	Aβ_{17-21}	-	100	>5.0	100
PPI-374	imino-biotinyl	Aβ_{16-20}	-	100	1.3	86
PPI-366	cholyl	Aβ_{15-19}	-	100	3.1	~0
				10	1.6	~0
PPI-369	cholyl	Aβ_{16-20} (Val$_{18}$->Thr)	-	100	~1	~0
PPI-370	cholyl	Aβ_{16-20} (Phe$_{19}$->Ala)	-	100	2.6	73
PPI-365	cholyl	(Ala)$_5$	-	100	~1	~0

Cb

PPI-319	_ cholyl	$A\beta_{16\text{-}20}$	amide	33	5.6	~0
				10	2.7	~0
PPI-321	cholyl	$A\beta_{16\text{-}19}$	amide	100	1.2	~0
PPI-377	-	$A\beta_{16\text{-}20}$	-	100	~1	~0

As shown in Table IV, cholyl-$A\beta_{16\text{-}20}$ (PPI-350) and cholyl-$A\beta_{17\text{-}21}$ (PPI-368) both exhibited inhibitory activity, indicating that the four-amino acid minimal subregion of positions 17-20 is sufficient for inhibitory activity. Loss of position 20 (e.g., in PPI-366 and PPI-321) resulted in loss of inhibitory activity, demonstrating the importance of position 20. Moreover, mutation of valine at position 18 to threonine (in PPI-369) also resulted in loss of activity, demonstrating the importance of position 18. In contrast, mutation of phenylalanine at position 19 to alanine (cholyl-$A\beta_{16\text{-}20}$ Phe_{19}->Ala; PPI-370) resulted in a compound which still retained detectable inhibitory activity. Accordingly, the phenylalanine at position 19 is more amenable to substitution, preferably with another hydrophobic amino acid residue. Cholyl-penta-alanine (PPI-365) showed no inhibitory activity, demonstrating the specificity of the $A\beta$ peptide portion of the modulator. Moreover, unmodified $A\beta_{16\text{-}20}$ (PPI-377) was not inhibitory, demonstrating the functional importance of the amino-terminal modifying group. The specific functional group influenced the activity of the modulator. For example, iminobiotinyl-$A\beta_{16\text{-}20}$ (PPI-374) exhibited inhibitory activity similar to cholyl-$A\beta_{16\text{-}20}$, whereas an N-acetyl neuraminic acid (NANA)-modified $A\beta_{16\text{-}20}$ was not an effective inhibitory modulator (not listed in Table IV). A C-terminal amide derivative of cholyl-$A\beta_{16\text{-}20}$ (PPI-319) retained high activity in delaying the lag time of aggregation, indicating that the carboxy-terminus of the modulator can be derivatized without loss of inhibitory activity. Although this amide-derivatized compound did not inhibit the overall plateau level of aggregation over time, the compound was not tested at concentrations higher than mole 33 %. Higher concentrations of the amide-derivatized compound are predicted to inhibit the overall plateau level of aggregation, similar to cholyl-$A\beta_{16\text{-}20}$ (PPI-350).

EXAMPLE 10:   **Effect of β-Amyloid Modulators on the Neurotoxicity of Natural β-Amyloid Peptide Aggregates**

The neurotoxicity of natural β-amyloid peptide aggregates, in either the presence or absence of a β-amyloid modulator, is tested in a cell-based assay using either a rat or human neuronally-derived cell line (PC-12 cells or NT-2 cells, respectively) and the viability indicator 3,(4,4-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT). (See e.g., Shearman, M.S. et al. (1994) Proc. Natl. Acad. Sci. USA 91:1470-1474; Hansen, M.B. et al. (1989) J. Immun. Methods 119:203-210 for a description of similar cell-based viability assays). PC-12 is a rat adrenal pheochromocytoma cell line and is available from the American Type Culture Collection, Rockville, MD (ATCC CRL 1721). MTT (commercially

available from Sigma Chemical Co.) is a chromogenic substrate that is converted from yellow to blue in viable cells, which can be detected spectrophotometrically.

To test the neurotoxicity of natural β-amyloid peptides, stock solutions of fresh Aβ monomers and aged Aβ aggregates were first prepared. $A\beta_{1-40}$ in 100% DMSO was
5   prepared from lyophilized powder and immediately diluted in one half the final volume in H_2O and then one half the final volume in 2X PBS so that a final concentration of 200 μM peptide, 4% DMSO is achieved. Peptide prepared in this way and tested immediately on cells is referred to as "fresh" Aβ monomer. To prepare "aged" Aβ aggregates, peptide solution was placed in a 1.5 ml Eppendorf tube and incubated at 37 °C for eight days to allow
10   fibrils to form. Such "aged" Aβ peptide can be tested directly on cells or frozen at -80°C. The neurotoxicity of fresh monomers and aged aggregates were tested using PC12 and NT2 cells. PC12 cells were routinely cultured in Dulbeco's modified Eagle's medium (DMEM) containing 10% horse serum, 5% fetal calf serum, 4mM glutamine, and 1% gentamycin. NT2 cells were routinely cultured in OPTI-MEM medium (GIBCO BRL CAT.
15   #31985) supplemented with 10% fetal calf serum, 2 mM glutamine and 1% gentamycin. Cells were plated at 10-15,000 cells per well in 90 μl of fresh medium in a 96 -well tissue culture plate 3-4 hours prior to treatment. The fresh or aged Aβ peptide solutions (10 μL) were then diluted 1:10 directly into tissue culture medium so that the final concentration was in the range of 1-10 μM peptide. Cells are incubated in the presence of peptide without a
20   change in media for 48 hours at 37°C. For the final three hours of exposure of the cells to the β-AP preparation, MTT was added to the media to a final concentration of 1 mg/ml and incubation was continued at 37 °C. Following the two hour incubation with MTT, the media was removed and the cells were lysed in 100 μL isopropanol/0.4N HCl with agitation. An equal volume of PBS was added to each well and the plates were agitated for an additional 10
25   minutes. Absorbance of each well at 570 nm was measured using a microtiter plate reader to quantitate viable cells.

The neurotoxicity of aged (5 day or 8 day) $A\beta_{1-40}$ aggregates alone, but not fresh $A\beta_{1-40}$ monomers alone, was confirmed in an experiment the results of which are shown in Figure 3, which demonstrates that incubating the neuronal cells with increasing amounts of
30   fresh $A\beta_{1-40}$ monomers was not significantly toxic to the cells whereas incubating the cells with increasing amounts of 5 day or 8 day $A\beta_{1-40}$ aggregates led to increasing amount of neurotoxicity. The EC50 for toxicity of aged $A\beta_{1-40}$ aggregates was 1-2 μM for both the PC12 cells and the NT2 cells.

To determine the effect of a β-amyloid modulator compound on the neurotoxicity of
35   $A\beta_{1-40}$ aggregates. a modulator compound, cholyl-$A\beta_{6-20}$ (PPI-264), was preincubated with $A\beta_{1-40}$ monomers under standard nucleation assay conditions as described in Example 6 and at particular time intervals post-incubation, aliquots of the β-AP/modulator solution were removed and 1) the turbidity of the solution was assessed as a measure of aggregation and 2) the solution was applied to cultured neuronal cells for 48 hours at which time cell viability

70

was assessed using MTT to determine the neurotoxicity of the solution. The results of the turbidity analysis are shown in Figure 4, panels A, B and C. In panel A, $A\beta_{1-40}$ and cholyl-$A\beta_{6-20}$ were both present at 64 μM. In panel B, $A\beta_{1-40}$ was present at 30 μM and cholyl-$A\beta_{6-20}$ was present at 64 μM. In panel C, $A\beta_{1-40}$ was present at 10 μM and cholyl-$A\beta_{6-20}$

5   was present at 64 μM. These data show that an equimolar amount of cholyl-$A\beta_{6-20}$ is effective at inhibiting aggregation of $A\beta_{1-40}$ (see Figure 4, panel A) and that as the concentration of $A\beta_{1-40}$ is reduced, the amount of detectable aggregation of the $A\beta_{1-40}$ monomer is correspondingly reduced (compare Figure 4, panels B and C with panel A). The corresponding results of the neurotoxicity analysis are shown in Figure 4, panels D, E, and F.

10   These results demonstrate that the β-amyloid modulator compound not only inhibits aggregation of $A\beta_{1-40}$ monomers but also inhibits the neurotoxicity of the $A\beta_{1-40}$ solution, illustrated by the reduced percent toxicity of the cells when incubated with the $A\beta_{1-40}$/modulator solution as compared to $A\beta_{1-40}$ alone (see *e.g.*, Figure 4, panel D). Moreover, even when $A\beta_{1-40}$ aggregation was not detectable as measured by light scattering,

15   the modulator compound inhibited the neurotoxicity of the $A\beta_{1-40}$ solution (see Figure 4, panels E and F). Thus, the formation of neurotoxic $A\beta_{1-40}$ aggregates precedes the formation of insoluble aggregates detectable by light scattering and the modulator compound is effective at inhibiting the inhibiting the formation and/or activity of these neurotoxic aggregates. Similar results were seen with other modulator compounds, such as

20   iminobiotinyl-$A\beta_{6-20}$ (PPI-267), cholyl-$A\beta_{16-20}$ (PPI-350) and cholyl-$A\beta_{16-20}$-amide (PPI-319).

        Additionally, the β-amyloid modulator compounds have been demonstrated to reduce the neurotoxicity of preformed $A\beta_{1-40}$ aggregates. In these experiments, $A\beta_{1-40}$ aggregates were preformed by incubation of the monomers in the absence of any modulators. The

25   modulator compound was then incubated with the preformed $A\beta_{1-40}$ aggregates for 24 hours at 37 °C, after which time the β-AP/modulator solution was collected and its neurotoxicity evaluated as described above. Incubation of preformed $A\beta_{1-40}$ aggregates with the modulator compound prior to applying the solution to neuronal cells resulted in a decrease in the neurotoxicity of the $A\beta_{1-40}$ solution. These results suggest that the modulator can either bind

30   to $A\beta$ fibrils or soluble aggregate and modulate their inherent neurotoxicity or that the modulator can perturb the equilibrium between monomeric and aggregated forms of $A\beta_{1-40}$ in favor of the non-neurotoxic form.

EXAMPLE 11:   **Characterization of Additional β-Amyloid Modulator Compounds**

35

        In this example, additional modulator compounds designed based upon amino acids 17-20 of $A\beta$, LVFF (identified in Example 9), were prepared and analyzed to further delineate the structural features necessary for inhibition of β-amyloid aggregation. Types of compounds analyzed included ones having only three amino acid residues of an $A\beta$

71

aggregation-core domain, compounds in which the amino acid residues of an Aβ aggregation
core domain were rearranged or in which amino acid substitutions had been made,
compounds modified with a carboxy-terminal modifying group and compounds in which the
modifying group had been derivatized. Abbreviations used in this example are: h- (free

5   amino terminus), -oh (free carboxylic acid terminus), -nh$_2$ (amide terminus), CA (cholyl, the
acyl portion of cholic acid), NANA (N-acetyl neuraminyl), IB (iminobiotinyl), βA (β-alanyl),
DA (D-alanyl), Adp (aminoethyldibenzofuranylpropanoic acid), Aic (3-(O-aminoethyl-iso)-
cholyl, a derivative of cholic acid), IY (iodotyrosyl), o-methyl (carboxy-terminal methyl
ester), N-me (N-methyl peptide bond), DeoxyCA (deoxycholyl) and LithoCA (lithocholyl).

10   Modulator compounds having an Aic modifying group at either the amino- or
carboxy-terminus (e.g., PPI-408 and PPI-418) were synthesized using known methods (see
e.g., Wess, G. et al. (1993) Tetrahedron Letters, 34:817-822; Wess, G. et al. (1992)
Tetrahedron Letters 33:195-198). Briefly, 3-iso-O-(2-aminoethyl)-cholic acid (3β-(2-
aminoethoxy)-7α,12α-dihydroxy-5β-cholanoic acid) was converted to the FMOC-protected

15 derivative using FMOC-OSu (the hydroxysuccinimide ester of the FMOC group, which is
commercially available) to obtain a reagent that was used to introduce the cholic acid
derivative into the compound. For N-terminal introduction of the cholic acid moiety, the
FMOC-protected reagent was coupled to the N-terminal amino acid of a solid-phase peptide
in the standard manner, followed by standard FMOC-deprotection conditions and subsequent

20 cleavage from the resin, followed by HPLC purification. For C-terminal introduction of the
cholic acid moiety, the FMOC-protected reagent was attached to 2-chlorotrityl chloride resin
in the standard manner. This amino acyl derivatized resin was then used in the standard
manner to synthesize the complete modified peptide.

           The modulators were evaluated in the nucleation and seeded extension assays
25 described in Example 6 and the results are summarized below in Table V. The change in lag
time (ΔLag) is presented as the ratio of the lag time observed in the presence of the test
compound to the lag time of the control. Data are reported for assays in the presence of 100
mole % inhibitor relative to the concentration of Aβ$_{1-40}$, except for PPI-315, PPI-348,
PPI-380, PPI-407 and PPI-418, for which the data is reported in the presence of 33 mole %

30 inhibitor. Inhibition (% I$_{nucl'n}$) is listed as the percent reduction in the maximum observed
turbidity in the control at the end of the assay time period. Inhibition in the extension assay
(% I$_{ext'n}$) is listed as the percent reduction of thioflavin-T fluorescence of β-structure in the
presence of 25 mole % inhibitor. Compounds with a % I$_{nucl'n}$ of at least 30% are highlighted
in bold.

72

Table V –

Ref. #	N-Term. Mod.	Peptide	C-Term. Mod.	ΔLag	% $I_{nucl'n}$	% $I_{ext'n}$
PPI-293	CA	-	-oh	1.0	0	ND*
PPI-315	CA	HQKLVFF	-nh₂	1.1	5**	ND
PPI-316	NANA	HQKLVFF	-nh₂	1.5	-15	ND
PPI-319	**CA**	**KLVFF**	**-nh₂**	**5.4**	**70**	**52**
PPI-339	CA	HDSGY	-nh₂	1.1	-18	ND
PPI-348	**CA**	**HQKLVFF**	**-oh**	**2.0**	**70****	**ND**
PPI-349	**CA**	**QKLVFF**	**-oh**	**>5**	**100**	**56**
PPI-350	**CA**	**KLVFF**	**-oh**	**1.8**	**72**	**11**
PPI-365	CA	AAAAA	-oh	0.8	-7	0
PPI-366	CA	QKLVF	-oh	3.1	-23	ND
PPI-368	**CA**	**LVFFA**	**-oh**	**>5**	**100**	**91**
PPI-369	CA	KLTFF	oh	1.1	-16	44
PPI-370	**CA**	**KLVAF**	**-oh**	**2.6**	**73**	**31**
PPI-371	**CA**	**KLVFF(βA)**	**-oh**	**2.5**	**76**	**80**
PPI-372	**CA**	**FKFVL**	**-oh**	**0.8**	**45**	**37**
PPI-373	NANA	KLVFF	-oh	0.9	16	8
PPI-374	**IB**	**KLVFF**	**-oh**	**1.3**	**86**	**0**
PPI-375	CA	KTVFF	-oh	1.2	18	21
PPI-377	h-	KLVFF	-oh	1.1	0	8
PPI-379	**CA**	**LVFFAE**	**-oh**	**1.4**	**55**	**16**
PPI-380	**CA**	**LVFF**	**-oh**	**1.8**	**72****	**51**
PPI-381	**CA**	**LVFF(DA)**	**-oh**	**2.3**	**56**	**11**
PPI-382	CA	LVFFA	-nh₂	1.0	-200	91
PPI-383	h-DDIIL-(Adp)	VFF	-oh	0.4	14	0
PPI-386	h-	LVFFA	-oh	1.0	15	11
PPI-387	h-	KLVFF	-nh₂	1.3	-9	39
PPI-388	**CA**	**AVFFA**	**-oh**	**1.4**	**68**	**44**
PPI-389	**CA**	**LAFFA**	**-oh**	**1.5**	**47**	**66**
PPI-390	CA	LVAFA	-oh	2.7	25	0
PPI-392	**CA**	**VFFA**	**-oh**	**2.0**	**76**	**10**
PPI-393	CA	LVF	-oh	1.3	1	0
PPI-394	**CA**	**VFF**	**-oh**	**1.8**	**55**	**0**
PPI-395	**CA**	**FFA**	**-oh**	**1.0**	**51**	**6**
PPI-396	**CA**	**LV(IY)FA**	**-oh**	**>5**	**100**	**71**
PPI-401	CA	LVFFA	-o-methyl	ND	ND	0
PPI-405	h-	LVFFA	-nh₂	1.3	11	70
PPI-407	**CA**	**LVFFK**	**-oh**	**>5**	**100****	**85**
PPI-408	**h-**	**LVFFA**	**(Aic)-oh**	**3.5**	**46**	**3**
PPI-418	**h-(Aic)**	**LVFFA**	**-oh**	**>5**	**100****	**87**
PPI-426	**CA**	**FFVLA**	**-oh**	**>5**	**100**	**89**
PPI-391	**CA**	**LVFAA**	**-oh**	**1.6**	**40**	**ND**
PPI-397	**CA**	**LVF(IY)A**	**-oh**	**>5**	**95**	**ND**
PPI-400	CA	AVAFA	-oh	1.0	-15	ND

73

PPI-403	–	***	HQKLVFF	-oh	1.4	-75	0
PPI-404		****	LKLVFF	-oh	1.8	-29	7
PPI-424	DeoxyCA		LVFFA	-oh	3.0	-114	82
PPI-425	LithoCA		LVFFA	-oh	2.8	-229	0
PPI-428	CA		FF	-oh	1.7	-78	15
PPI-429	CA		FFV	-oh	2.2	-33	7
PPI-430	**CA**		**FFVL**	**-oh**	**4.1**	**33**	**75**
PPI-433	CA		LVFFA (all D amino acids)	-oh	2.8	27	ND
PPI-435	t-Boc		LVFFA	-oh	3.0	-5	ND
PPI-438	CA		GFF	-oh	1.0	0	ND

* ND = not done
** = 33 mol %
*** = h-DDIII(N-Me-Val)DLL(Adp)
****= h-DDII(N-Me-Leu)VEH(Adp)

Certain compounds shown in Table V (PPI-319, PPI-349, PPI-350, PPI-368 and PPI-426) also were tested in neurotoxicity assays such as those described in Example 10. For each compound, the delay of the appearance of neurotoxicity relative to control coincided with the delay in the time at which polymerization of Aβ began in the nucleation assays. This correlation between the prevention of formation of neurotoxic Aβ species and the prevention of polymerization of Aβ was consistently observed for all compounds tested.

The results shown in Table V demonstrate that at an effective modulator compound can comprise as few as three Aβ amino acids residues (see PPI-394, comprising the amino acid sequence VFF, which corresponds to Aβ$_{18-20}$, and PPI-395, comprising the amino acid sequence FFA, which corresponds to Aβ$_{19-21}$). The results also demonstrate that a modulator compound having a modulating group at its carboxy-terminus is effective at inhibiting Aβ aggregation (see PPI-408, modified at its C-terminus with Aic). Still further, the results demonstrate that the cholyl group, as a modulating group, can be manipulated while maintaining the inhibitory activity of the compounds (see PPI-408 and PPI-418, both of which comprise the cholyl derivative Aic). The free amino group of the Aic derivative of cholic acid represents a position at which a chelation group for ^{99m}Tc can be introduced, e.g., to create a diagnostic agent. Additionally, the ability to substitute iodotyrosyl for phenylalanine at position 19 or 20 of the Aβ sequence (see PPI-396 and PPI-397) while maintaining the ability of the compound to inhibit Aβ aggregation indicates that the compound could be labeled with radioactive iodine, e.g., to create a diagnostic agent, without loss of the inhibitory activity of the compound.

Finally, compounds with inhibitory activity were created using Aβ derived amino acids but wherein the amino acid sequence was rearranged or had a substitution with a non-Aβ-derived amino acid. Examples of such compounds include PPI-426, in which the sequence of Aβ$_{17-21}$ (LVFFA) has been rearranged (FFVLA), PPI-372, in which the

74

sequence of Aβ$_{16-20}$ (KLVFF) has been rearranged (FKFVL), and PPI-388, -389 and -390, in which the sequence of Aβ$_{17-21}$ (LVFFA) has been substituted at position 17, 18 or 19, respectively, with an alanine residue (AVFFA for PPI-388, LAFFA for PPI-389 and LVAFA for PPI-390). The inhibitory activity of these compounds indicate that the presence in the compound of an amino acid sequence directly corresponding to a portion of Aβ is not essential for inhibitory activity, but rather suggests that maintenance of the hydrophobic nature of this core region, by inclusion of amino acid residues such as phenylalanine, valine, leucine, regardless of their precise order, can be sufficient for inhibition of Aβ aggregation.

EXAMPLE 12:   Characterization of β-Amyloid Modulator Compounds Comprising an Unmodified β-Amyloid Peptide

To examine the ability of unmodified Aβ peptides to modulate aggregation of natural β-AP, a series of Aβ peptides having amino- and/or carboxy terminal deletions as compared to Aβ$_{1-40}$, or having internal amino acids deleted (*i.e.*, noncontiguous peptides), were prepared. One peptide (PPI-220) had additional, non-Aβ-derived amino acid residues at its amino-terminus. These peptides all had a free amino group at the amino-terminus and a free carboxylic acid at the carboxy-terminus. These unmodified peptides were evaluated in assays as described in Example 7. The results are summarized below in Table VI, wherein the data is presented as described in Example 7. Compounds exhibiting at least a 1.5 fold increase in lag time are highlighted in bold.

Table VI

Reference #	Aβ Peptide	Mole %	Fold Increase in Lag Time	% Inhibition of Plateau
PPI-226	**Aβ$_{6-20}$**	**100**	**1.66**	**76**
PPI-227	Aβ$_{11-25}$	100	~1	47
PPI-228	**Aβ$_{16-30}$**	**100**	**>4.5**	**100**
PPI-229	Aβ$_{21-35}$	100	~1	~0
PPI-230	Aβ$_{26-40}$	100	0.8	~0
PPI-231	Aβ$_{1-15}$	100	~1	18
PPI-247	Aβ$_{1-30, 36-40}$ (Δ31-35)	100	~1	~0
PPI-248	**Aβ$_{1-25, 31-40}$ (Δ26-30)**	**100**	**1.58**	**~0**
PPI-249	**Aβ$_{1-20, 26-40}$ (Δ21-25)**	**100**	**2.37**	**~0**
PPI-250	**Aβ$_{1-15, 21-40}$ (Δ16-20)**	**100**	**1.55**	**~0**
PPI-251	Aβ$_{1-10, 16-40}$ (Δ11-15)	100	~1.2	~0
PPI-252	**Aβ$_{1-5, 11-40}$ (Δ6-10)**	**100**	**1.9**	**33**
PPI-253	**Aβ$_{6-40}$**	**100**	**1.9**	**~0**
PPI-220	**EEVVHHHHQQ-Aβ$_{16-40}$**	**100**	**>4**	**100**

75

The results shown in Table VI demonstrate that limited portions of the Aβ sequence can have a significant inhibitory effect on natural β-AP aggregation even when the peptide is not modified by a modifying group. Preferred unmodified peptides are Aβ$_{6-20}$ (PPI-226), Aβ$_{16-30}$ (PPI-228), Aβ$_{1-20, 26-40}$ (PPI-249) and EEVVHHHHQQ-Aβ$_{16-40}$ (PPI-220), the amino acid sequences of which are shown in SEQ ID NOs: 4, 14, 15, and 16, respectively.

Forming part of this disclosure is the appended Sequence Listing, the contents of which are summarize in the Table below.

SEQ ID NO:	Amino Acids	Peptide Sequence
1	43 amino acids	Aβ$_{1-43}$
2	103 amino acids	APP C-terminus
3	43 amino acids	Aβ$_{1-43}$ (19. 20 mutated)
4	HDSGYEVHHQKLVFF	Aβ$_{6-20}$
5	HQKLVFFA	Aβ$_{14-21}$
6	HQKLVFF	Aβ$_{14-20}$
7	QKLVFFA	Aβ$_{15-21}$
8	QKLVFF	Aβ$_{15-20}$
9	KLVFFA	Aβ$_{16-21}$
10	KLVFF	Aβ$_{16-20}$
11	LVFFA	Aβ$_{17-21}$
12	LVFF	Aβ$_{17-20}$
13	LAFFA	Aβ$_{17-21}$ (V$_{18}$→A)
14	KLVFFAEDVGSNKGA	Aβ$_{16-30}$
15	35 amino acids	Aβ$_{1-20, 26-40}$
16	35 amino acids	EEVVHHHHQQ-βAP$_{16-40}$
17	AGAAAAGA	PrP peptide
18	AILSS	amylin peptide
19	VFF	Aβ$_{18-20}$
20	FFA	Aβ$_{19-21}$
21	FFVLA	Aβ$_{17-21}$ (scrambled)
22	LVFFK	Aβ$_{17-21}$ (A$_{21}$→K)
23	LV(IY)FA	Aβ$_{17-21}$ (F$_{19}$→IY)
24	VFFA	Aβ$_{18-21}$
25	AVFFA	Aβ$_{17-21}$ (L$_{17}$→A)
26	LVF(IY)A	Aβ$_{17-21}$ (F$_{20}$→IY)
27	LVFFAE	Aβ$_{17-22}$
28	FFVL	Aβ$_{17-20}$ (scrambled)

76

29 –	FKFVL	$A\beta_{16-20}$ (scrambled)
30	KLVAF	$A\beta_{16-20}$ ($F_{19} \rightarrow A$)
31	KLVFF(βA)	$A\beta_{16-21}$ ($A_{21} \rightarrow \beta A$)
32	LVFF(DA)	$A\beta_{17-21}$ ($A_{21} \rightarrow DA$)

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5   (i) APPLICANT:
              (A) NAME: PHARMACEUTICAL PEPTIDES INCORPORATED
              (B) STREET: ONE HAMPSHIRE STREET
              (C) CITY: CAMBRIDGE
              (D) STATE: MASSACHUSETTS
10   (E) COUNTRY: USA
              (F) POSTAL CODE (ZIP): 02139-1572
              (G) TELEPHONE: (617) 494-8400
              (H) TELEFAX: (617) 494-8414

15   (ii) TITLE OF INVENTION: Modulators of Amyloid Aggregation

         (iii) NUMBER OF SEQUENCES: 32

         (iv) CORRESPONDENCE ADDRESS:
20   (A) ADDRESSEE: LAHIVE & COCKFIELD
              (B) STREET: 60 State Street, Suite 510
              (C) CITY: Boston
              (D) STATE: Massachusetts
              (E) COUNTRY: USA
25   (F) ZIP: 02109-1875

          (v) COMPUTER READABLE FORM:
              (A) MEDIUM TYPE: Floppy disk
              (B) COMPUTER: IBM PC compatible
30   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
              (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

         (vi) CURRENT APPLICATION DATA:
              (A) APPLICATION NUMBER: 000000
35   (B) FILING DATE: Herewith
              (C) CLASSIFICATION:

        (vii) PRIOR APPLICATION DATA:
              (A) APPLICATION NUMBER: USSN 08/404,831
40   (B) FILING DATE: 14-MAR-1995

        (vii) PRIOR APPLICATION DATA:
              (A) APPLICATION NUMBER: USSN 08/475,579
              (B) FILING DATE: 07-JUN-1995
45
        (vii) PRIOR APPLICATION DATA:
              (A) APPLICATION NUMBER: USSN 08/548,998
              (B) FILING DATE: 27-OCT-1995

50   (viii) ATTORNEY/AGENT INFORMATION:
              (A) NAME: DeConti, Giulio A.
              (B) REGISTRATION NUMBER: 31,503
              (C) REFERENCE/DOCKET NUMBER: PPI-002C2PC

55   (ix) TELECOMMUNICATION INFORMATION:
              (A) TELEPHONE: (617)227-7400
              (B) TELEFAX: (617)227-5941

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 43 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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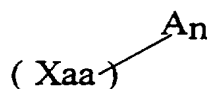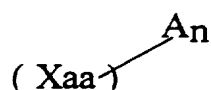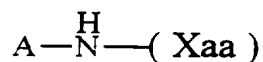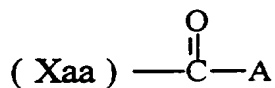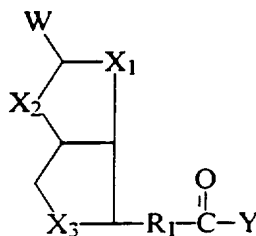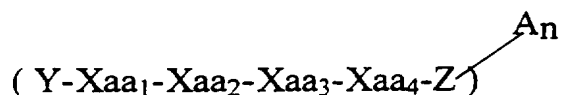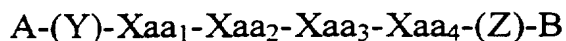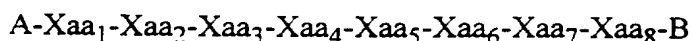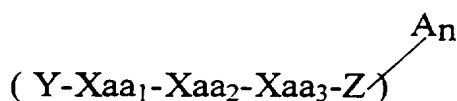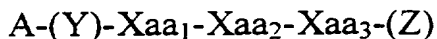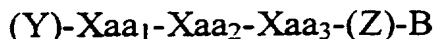Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1               5                   10              15

Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
            20                  25                  30

Gly Leu Met Val Gly Gly Val Val Ile Ala Thr
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 103 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (v) FRAGMENT TYPE: internal

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
1               5                   10              15

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
            20                  25                  30

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
            35                  40                  45

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
        50                  55                  60

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
65                  70                  75                  80

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
                85                  90                  95

Phe Phe Glu Gln Met Gln Asn
                100
```

(2) INFORMATION FOR SEQ ID NO:3:

```
     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 43 amino acids
         (B) TYPE: amino acid
 5       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

     (v) FRAGMENT TYPE: internal
10
    (ix) FEATURE:
         (A) NAME/KEY: Modified site
         (B) LOCATION: 19
         (D) OTHER INFORMATION: /note= Xaa is a hydrophobic amino
15                                       acid

    (ix) FEATURE:
         (A) NAME/KEY: Modified site
         (B) LOCATION: 20
20       (D) OTHER INFORMATION: /note= Xaa is a hydrophobic amino
                                         acid

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

25  Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
    1               5                   10                  15

    Leu Val Xaa Xaa Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
                20                   25                  30
30
    Gly Leu Met Val Gly Gly Val Val Ile Ala Thr
            35                  40


35  (2) INFORMATION FOR SEQ ID NO:4:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 15 amino acids
         (B) TYPE: amino acid
40       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
45
    His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe
                    5                   10                  15


50  (2) INFORMATION FOR SEQ ID NO:5:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 8 amino acids
         (B) TYPE: amino acid
55       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
```

80

```
      His Gln Lys Leu Val Phe Phe Ala
                     5
```

5

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7 amino acids

10
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

15   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
      His Gln Lys Leu Val Phe Phe
                     5
```

20

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7 amino acids

25
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

30   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
      Gln Lys Leu Val Phe Phe Ala
                     5
```

35

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6 amino acids

40
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

45   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
      Gln Lys Leu Val Phe Phe
                     5
```

50

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6 amino acids

55
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

81

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Leu Val Phe Phe Ala
                      5

5

(2) INFORMATION FOR SEQ ID NO:10:

10
            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 5 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: peptide
15
            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Leu Val Phe Phe
                    5
20

(2) INFORMATION FOR SEQ ID NO:11:

            (i) SEQUENCE CHARACTERISTICS:
25   (A) LENGTH: 5 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: peptide
30
            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Val Phe Phe Ala
                  5
35

(2) INFORMATION FOR SEQ ID NO:12:

            (i) SEQUENCE CHARACTERISTICS:
40   (A) LENGTH: 4 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: peptide
45
            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Val Phe Phe

50

(2) INFORMATION FOR SEQ ID NO:13:

            (i) SEQUENCE CHARACTERISTICS:
55   (A) LENGTH: 5 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: peptide

82

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Ala Phe Phe Ala
1   5

(2) INFORMATION FOR SEQ ID NO:14:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 15 amino acids
           (B) TYPE: amino acid
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: peptide

      (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala
1   5   10   15

(2) INFORMATION FOR SEQ ID NO:15:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 35 amino acids
           (B) TYPE: amino acid
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: peptide

      (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1   5   10   15

Leu Val Phe Phe Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val Gly
            20   25   30

Gly Val Val
            35

(2) INFORMATION FOR SEQ ID NO:16:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 35 amino acids
           (B) TYPE: amino acid
           (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Glu Glu Val Val His His His His Gln Gln Lys Leu Val Phe Phe Ala
1   5   10   15

Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val Gly
            20   25   30

Gly Val Val
        35

(2) INFORMATION FOR SEQ ID NO:17:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 8 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (v) FRAGMENT TYPE: internal

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

        Ala Gly Ala Ala Ala Ala Gly Ala
        1   5

(2) INFORMATION FOR SEQ ID NO:18:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 5 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (v) FRAGMENT TYPE: internal

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

        Ala Ile Leu Ser Ser
        1   5

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 3 amino acids
                (B) TYPE: amino acid
                (D) TOPOLOGY: linear

84

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Val Phe Phe
1

(2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 3 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Phe Ala
1

(2) INFORMATION FOR SEQ ID NO:21:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Phe Phe Val Leu Ala
1   5

(2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Val Phe Phe Lys
1   5

(2) INFORMATION FOR SEQ ID NO:23:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid

         (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

5   (ix) FEATURE:
      (A) NAME/KEY: Modified site
      (B) LOCATION: 3
      (D) OTHER INFORMATION: /note= Xaa is iodotyrosyl

10   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Leu Val Xaa Phe Ala
1   5

15

(2) INFORMATION FOR SEQ ID NO:24:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 4 amino acids
20   (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

25   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Val Phe Phe Ala
1

30

(2) INFORMATION FOR SEQ ID NO:25:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 5 amino acids
35   (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

40   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala Val Phe Phe Ala
1   5

45

(2) INFORMATION FOR SEQ ID NO:26:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 5 amino acids
50   (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

55   (ix) FEATURE:
      (A) NAME/KEY: Modified site
      (B) LOCATION: 4
      (D) OTHER INFORMATION: /note= Xaa is iodotyrosyl

4u

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Leu Val Phe Xaa Ala
1

5

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
10   (A) LENGTH: 6 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide
15
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

    Leu Val Phe Phe Ala Glu
    1   5
20

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
25   (A) LENGTH: 4 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide
30
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

    Phe Phe Val Leu
    1   5
35

(2) INFORMATION FOR SEQ ID NO:29:

    (i) SEQUENCE CHARACTERISTICS:
40   (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide
45
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

    Phe Lys Phe Val Leu
    1   5
50

(2) INFORMATION FOR SEQ ID NO:30:

    (i) SEQUENCE CHARACTERISTICS:
55   (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

87

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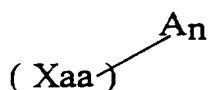(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

    Lys Leu Val Ala Phe
    1               5


    (2) INFORMATION FOR SEQ ID NO:31:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 6 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: peptide

       (ix) FEATURE:
            (A) NAME/KEY: Modified site
            (B) LOCATION: 6
            (D) OTHER INFORMATION: /note= Xaa is beta-alanyl

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

    Lys Leu Val Phe Phe Xaa
    1


    (2) INFORMATION FOR SEQ ID NO:32:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 5 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: peptide

       (ix) FEATURE:
            (A) NAME/KEY: Modified site
            (B) LOCATION: 5
            (D) OTHER INFORMATION: /note= Xaa is D-alanyl

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

    Leu Val Phe Phe Xaa
    1
```

CLAIMS

1.   An amyloid modulator compound comprising an amyloidogenic protein, or peptide fragment thereof, coupled directly or indirectly to at least one modifying group such that the compound modulates the aggregation of natural amyloid proteins or peptides when contacted with the natural amyloidogenic proteins or peptides.

2.   The compound of claim 1, which inhibits aggregation of natural amyloidogenic proteins or peptides when contacted with the natural amyloidogenic proteins or peptides.

3.   The compound of claim 1, wherein the amyloidogenic protein, or peptide fragment thereof, is selected from the group consisting of transthyretin (TTR), prion protein (PrP), islet amyloid polypeptide (IAPP), atrial natriuretic factor (ANF), kappa light chain, lambda light chain, amyloid A, procalcitonin, cystatin C, β2 microglobulin, ApoA-I, gelsolin, procalcitonin, calcitonin, fibrinogen and lysozyme.

4.   A β-amyloid peptide compound comprising a formula:

$$(Xaa)\overset{An}{\diagup}$$

wherein Xaa is a β-amyloid peptide having an amino-terminal amino acid residue corresponding to position 668 of β-amyloid precursor protein-770 (APP-770) or to a residue carboxy-terminal to position 668 of APP-770. A is a modifying group attached directly or indirectly to the β-amyloid peptide of the compound such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. and n is an integer selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

5.   The compound of claim 4, wherein at least one A group is attached directly or indirectly to the amino terminus of the β-amyloid peptide of the compound.

6.   The compound of claim 4, wherein at least one A group is attached directly or indirectly to the carboxy terminus of the β-amyloid peptide of the compound.

7.   The compound of claim 4, wherein at least one A group is attached directly or indirectly to a side chain of at least one amino acid residue of the β-amyloid peptide of the compound.

8.   A β-amyloid modulator compound comprising an Aβ aggregation core domain (ACD) coupled directly or indirectly to at least one modifying group (MG) such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid

5   peptides when contacted with the natural β-amyloid peptides.

9.   The compound of claim 8, wherein the Aβ aggregation core domain is modeled after a subregion of natural β-amyloid peptide between 3 and 10 amino acids in length.

10

10.   A β-amyloid modulator compound comprising a formula:

$$(Y-Xaa_1-Xaa_2-Xaa_3-Z)^{An}$$

15   wherein Xaa_1. Xaa_2 and Xaa_3 are each amino acid structures and at least two of Xaa_1, Xaa_2 and Xaa_3 are, independently, selected from the group consisting of a leucine structure, a phenylalanine structure and a valine structure;

Y, which may or may not be present, is a peptidic structure having the formula $(Xaa)_a$, wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

20   Z, which may or may not be present, is a peptidic structure having the formula $(Xaa)_b$, wherein Xaa is any amino acid structure and b is an integer from 1 to 15; and

A is a modifying group attached directly or indirectly to the compound and n is an integer;

Xaa_1. Xaa_2. Xaa_3. Y, Z, A and n being selected such that the compound modulates

25   the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

11.   The compound of claim 10, wherein Xaa_1 and Xaa_2 are each phenylalanine structures.

30

12.   The compound of claim 10, wherein Xaa_2 and Xaa_3 are each phenylalanine structures.

13.   A β-amyloid modulator compound comprising a formula:

35

$$(Y-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Z)^{An}$$

wherein Xaa$_1$ and Xaa$_3$ are amino acid structures;

Xaa$_2$ is a valine structure;

Xaa$_4$ is a phenylalanine structure;

Y, which may or may not be present, is a peptidic structure having the formula (Xaa)$_a$, wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

Z, which may or may not be present, is a peptidic structure having the formula (Xaa)$_b$, wherein Xaa is any amino acid structure and b is an integer from 1 to 15; and

A is a modifying group attached directly or indirectly to the compound and n is an integer;

Xaa$_1$, Xaa$_3$, Y, Z, A and n being selected such that the compound modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

14.   The compound of claim 13, wherein Xaa$_1$ is a leucine structure and Xaa$_3$ is phenylalanine structure.

15.   A compound comprising the formula:

A-Xaa$_1$-Xaa$_2$-Xaa$_3$-Xaa$_4$-Xaa$_5$-Xaa$_6$-Xaa$_7$-Xaa$_8$-B

wherein Xaa1 is a histidine structure;

Xaa2 is a glutamine structure;

Xaa3 is a lysine structure;

Xaa4 is a leucine structure;

Xaa5 is a valine structure;

Xaa6 is a phenylalanine structure;

Xaa7 is a phenylalanine structure;

Xaa8 is an alanine structure;

A and B are modifying groups attached directly or indirectly to the amino terminus and carboxy terminus, respectively, of the compound;

and wherein Xaa$_1$-Xaa$_2$-Xaa$_3$, Xaa$_1$-Xaa$_2$ or Xaa$_1$ may or may not be present;

Xaa$_8$ may or may not be present; and

at least one of A and B is present.

16.   A β-amyloid modulator compound comprising a modifying group attached directly or indirectly to a peptidic structure, wherein the peptidic structure comprises amino acid structures having an amino acid sequence selected from the group consisting of His-Gln-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 5), His-Gln-Lys-Leu-Val-Phe-Phe (SEQ ID NO: 6),

91

Gln-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 7), Gln-Lys-Leu-Val-Phe-Phe (SEQ ID NO: 8),
Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO: 9), Lys-Leu-Val-Phe-Phe (SEQ ID NO: 10), Leu-
Val-Phe-Phe-Ala (SEQ ID NO: 11), Leu-Val-Phe-Phe (SEQ ID NO: 12), Leu-Ala-Phe-Phe-
Ala (SEQ ID NO: 13), Val-Phe-Phe (SEQ ID NO: 19), Phe-Phe-Ala (SEQ ID NO: 20), Phe-
Phe-Val-Leu-Ala (SEQ ID NO: 21), Leu-Val-Phe-Phe-Lys (SEQ ID NO: 22), Leu-Val-
Iodotyrosine-Phe-Ala (SEQ ID NO: 23), Val-Phe-Phe-Ala (SEQ ID NO: 24), Ala-Val-Phe-
Phe-Ala (SEQ ID NO: 25), Leu-Val-Phe-Iodotyrosine-Ala (SEQ ID NO: 26), Leu-Val-Phe-
Phe-Ala-Glu (SEQ ID NO: 27), Phe-Phe-Val-Leu (SEQ ID NO: 28), Phe-Lys-Phe-Val-Leu
(SEQ ID NO: 29), Lys-Leu-Val-Ala-Phe (SEQ ID NO: 30), Lys-Leu-Val-Phe-Phe-βAla
(SEQ ID NO: 31) and Leu-Val-Phe-Phe-DAla (SEQ ID NO: 32).

17.   The compound of claim 1-16, wherein the modifying group comprises a
cyclic, heterocyclic or polycyclic group.

18.   The compound of claim 1-16, wherein the modifying group contains a cis-
decalin group.

19.   The compound of claim 1-16, wherein the modifying group contains a
cholanoyl structure.

20.   The compound of claim 1-16, wherein the modifying group is a cholyl group.

21.   The compound of claim 1-16, wherein the modifying group comprises a
biotin-containing group, a diethylene-triaminepentaacetyl group, a (-)-menthoxyacetyl group,
a fluorescein-containing group or an N-acetylneuraminyl group.

22.   The compound of claim 1-16, which is further modified to alter a
pharmacokinetic property of the compound.

23.   The compound of claim 1-16, which is further modified to label the compound
with a detectable substance.

24.   The β-amyloid modulator which inhibits aggregation of natural β-amyloid
peptides when contacted with a molar excess amount of natural β-amyloid peptides.

25.   A β-amyloid peptide compound comprising an amino acid sequence having at
least one amino acid deletion compared to βAP_{1-39}, such that the compound inhibits
aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid
peptides.

92

26.   The compound of claim 25, comprising an amino acid sequence which has at least one internal amino acid deleted compared to βAP_{1-39}.

5

27.   The compound of claim 25, comprising an amino acid sequence which has at least one N-terminal amino acid deleted compared to βAP_{1-39}.

10   28.   The compound of claim 25, comprising an amino acid sequence which has at least one C-terminal amino acid deleted compared to βAP_{1-39}.

29.   A compound selected from the group consisting of βAP_{6-20} (SEQ ID NO: 4), βAP_{16-30} (SEQ ID NO: 14), $\beta AP_{1-20, 26-40}$ (SEQ ID NO: 15) and EEVVHHHHQQ-βAP_{16-40} (SEQ ID NO: 16).

15

30.   A pharmaceutical composition comprising the compound of claim 1-29 and a pharmaceutically acceptable carrier.

20   31   Use of the compound of claim 1-29 in the manufacture of a medicament for the diagnosis of an amyloidogenic disease.

32.   Use of the compound of claim 1-29 in the manufacture of a medicament for the treatment of an amyloidogenic disease.

25

33.   A method for inhibiting aggregation of natural β-amyloid peptides, comprising contacting the natural β-amyloid peptides with the compound of claims 4-29 such that aggregation of the natural β-amyloid peptides is inhibited.

30   34.   A method for inhibiting neurotoxicity of natural β-amyloid peptides, comprising contacting the natural β-amyloid peptides with the compound of claim 4-29 such that neurotoxicity of the natural β-amyloid peptides is inhibited.

35.   A method for detecting the presence or absence of natural β-amyloid peptides

35   in a biological sample. comprising:
            contacting a biological sample with the compound of 4-29; and
            detecting the compound bound to natural β-amyloid peptides to thereby detect the presence or absence of natural β-amyloid peptides in the biological sample.

95

36.   ⁻ The method of claim 35, wherein the β-amyloid modulator compound and the biological sample are contacted *in vitro*.

37.   The method of claim 35, wherein the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject.

38.   The method of claim 37, wherein the compound is labeled with radioactive technetium or radioactive iodine.

39.   A method for detecting natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease, comprising:
        contacting a biological sample with the compound of claim 4-29; and
        detecting the compound bound to natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease.

40.   The method of claim 39, wherein the β-amyloid modulator compound and the biological sample are contacted *in vitro*.

41.   The method of claim 39, wherein the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject.

42.   The method of claim 41, wherein the compound is labeled with radioactive technetium or radioactive iodine.

43.   The method of claim 39, which facilitates diagnosis of Alzheimer's disease.

44.   A method for treating a subject for a disorder associated with amyloidosis, comprising:
        administering to the subject a therapeutically or prophylactically effective amount of the compound of claim 1-3 such that the subject is treated for a disorder associated with amyloidosis.

45.   The method of claim 44, wherein the disorder is selected from the group consisting of familial amyloid polyneuropathy (Portuguese, Japanese and Swedish types), familial amyloid cardiomyopathy (Danish type), isolated cardiac amyloid, systemic senile amyloidosis, scrapie, bovine spongiform encephalopathy, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, adult onset diabetes, insulinoma, isolated atrial

94

amyloidosis, idiopathic (primary) amyloidosis, myeloma or macroglobulinemia-associated amyloidosis, primary localized cutaneous nodular amyloidosis associated with Sjogren's syndrome, reactive (secondary) amyloidosis, familial Mediterranean Fever and familial amyloid nephropathy with urticaria and deafness (Muckle-Wells syndrome), hereditary
5   cerebral hemorrhage with amyloidosis of Icelandic type, amyloidosis associated with long term hemodialysis, hereditary non-neuropathic systemic amyloidosis (familial amyloid polyneuropathy III), familial amyloidosis of Finnish type, amyloidosis associated with medullary carcinoma of the thyroid, fibrinogen-associated hereditary renal amyloidosis and lysozyme-associated hereditary systemic amyloidosis.
10

46.   A method for treating a subject for a disorder associated with β-amyloidosis, comprising:
    administering to the subject a therapeutically effective amount of the compound of claim 4-29 such that the subject is treated for a disorder associated with β-amyloidosis.
15

47.   The method of claim 46, wherein the disorder is Alzheimer's disease.

48.   A method for treating a subject for a disorder associated with β-amyloidosis, comprising:
20   administering to the subject a recombinant expression vector encoding the compound of claim 25-29 such that the compound is synthesized in the subject and the subject is treated for a disorder associated with β-amyloidosis.

49.   The method of claim 48, wherein the disorder is Alzheimer's disease.
25

Aggregation of $A\beta_{1-40}$ in the presence of N-biotinyl-$A\beta_{1-40}$

$\Delta A_{400\,nm}$

Time (h)

Legend:
- ● — Control
- ■ · 1% N-biotinyl-$A\beta_{1-40}$
- △ · 5% N-biotinyl-$A\beta_{1-40}$

Aggregation Assay $A\beta_{1-40}$

Increase in turbidity measured as the change in absorbance at 400nm: ● Control, 64μM Aβ alone; ■ 1mole% N-Biotinyl-$A\beta_{1-40}$ added; △ 5mol% N-Biotinyl-$A\beta_{1-40}$ added.

FIGURE 1

2-Iminobiotin-NHS ester

Diethylenetriaminepentaacetic
Dianhydride

4-Morpholinecarbonyl Chloride

2-Thiopheneacetyl Chloride

2-Thiophenesulfonyl Chloride

Fluorescein Isothiocyanate
Isomer I

5-(and-6)-Carboxyfluorescein,
succinimidyl ester

N-Acetylneuraminic Acid

Cholic Acid

trans-4-Cotininecarboxylic Acid

2-Imino-1-
Imidazolidineacetic Acid

(S)-(-)-Indoline-2-
carboxylic Acid

(-)-Menthoxyacetic Acid

2-Norbornaneacetic Acid

γ-Oxo-5-acenaphthenebutyric Acid

(-)-2-Oxo-4-thiazolidine-
carboxylic Acid

Tetrahydro-3-furoic Acid

FIGURE 2

FIGURE 3

FIGURE 4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C07K14/47   A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 28412 (MIRIAM HOSPITAL) 8 December 1994 see the whole document ---	1-16,23, 30,31, 35-43
X	WO,A,93 04194 (UNIV MINNESOTA ;HARVARD COLLEGE (US)) 4 March 1993 see the whole document --- -/--	1,2, 4-21,23, 24,30, 31,35-43

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 1996	2 8. 08. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Groenendijk, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.MOL.BIOL., vol. 228, 1992, pages 460-473, XP002010983 C.HILBICH E.A.: "Substitutions of hydrophobic amino acids reduce the amyloidogenicity of Alzheimer's disease beta-A4 peptides" cited in the application see the whole document ---	1,2, 4-13,16, 24,25, 27,30, 32-34, 44-49
X	BIOCHEMISTRY, vol. 34, no. 3, 24 January 1995, EASTON, PA US, pages 724-730, XP002010984 S.J.WOOD E.A.: "Prolines and amyloidogenicity in fragments of the Alzheimer's peptide beta/A4" see the whole document ---	1,2,4-6, 8-17,24
X	SOCIETY FOR NEUROSCIENCE ABSTRACTS, VOL.19 NO.1-3, 1993, P.861 XP002011313 see page 861 ---	1,2,17, 21-23
X	CHEMICAL ABSTRACTS, vol. 119, no. 3, 19 July 1993 Columbus, Ohio, US; abstract no. 23024, H.INOUYE E.A.: "Structure of beta-crystalline assemblies formed by beta-amyloid protein analogs by x-ray diffraction" page 349; column 2; XP002010988 see abstract & BIOPHYS J., vol. 64, no. 2, 1993, pages 502-519, ---	24,25, 27,28
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, January 1994, WASHINGTON US, pages 380-384, XP002010985 J.F.FLOOD E.A.: "Topography of a binding site for small amnestic peptides deduced from structure-activity studies" see the whole document ---	24,25, 27,28,30
X	J.A.SMITH E.A.: "Peptides,Chemistry and Biology; Proc. 12th Am. Peptide Symp.1991" 1992 , ESCOM , LEIDEN XP002010987 S.B.Vyas e.a.:"Characterisation of aggregation in Alzheimer beta-protein ... see page 278 - page 279 ---	24,25, 27,28

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP,A,0 681 844 (KANEGAFUCHI KAGAKU KOGYO KABUSHIKI KAISHA) 15 November 1995 see the whole document ---	1-3,21, 23,31
P,X	WO,A,95 12815 (UNIV NEW YORK) 11 May 1995 see the whole document ---	1,2,30, 32,44,45
P,X	WO,A,95 20979 (PICOWER INST MED RES ;VITEK MICHAEL P (US); CERAMI ANTHONY (US); B) 10 August 1995 see the whole document ---	1,2, 8-17,30
T	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 15, 12 April 1996, MD US, pages 8545-8548, XP002010986 L.O.TJERNBERG E.A.: "Arrest of beta-amyloid fibril formation by a pentapeptide ligand" see the whole document ---	24-49
A	EP,A,0 554 887 (KREMERS URBAN CO) 11 August 1993 see the whole document -----	1-49

1

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

   See Further Information sheet enclosed.

   — see "Remark" —

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Remark : Although claims 33-35, 37-39 and 41-49 as far as related to in vivo
methods are directed to a method of treatment of (diagnostic method
practised on) the human/animal body the search has been carried out
and based on the alleged effects of the compound/composition.

OBSCURITIES

The scope of the claims 1-13 and 17-21 is very broad and speculative.
Compounds which are mainly, and in at least part of the claims even
exclusively, defined by expressions like "amyloidogenic protein" and
"modifying group", which expressions themselves are ill-defined, cannot
be considered to represent a clear and concise definition of patentable
subject matter (Art.6 PCT)
Furthermore the available experimental data actually only comprise a very
small part of the compounds claimed, which part is moreover not evenly
distributed over the whole claimed area (actually only specifically modified
fragments of beta-amyloid protein have been exemplified). Therefore the claims
can also not be considered to represent a permissible generalization which is
fairly based on experimental evidence, that is, they are also not adequately
supported by the description (Art.6 PCT)
Therefore a meaningful and economically feasible search could not encompass
the complete subject-matter of the claims.
Consequently the search has been limited to the actually synthesized examples
and (closely) related analogs and therefore only the claims 14-16 and 25-29
have been searched completely. (Art.17(2)(a)(ii)PCT)

Claims searched completely :   14-16, 25-29.
Claims searched incompletely : 1, 2, 4-13, 17-24, 30-49
Claims not searched :   3

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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WO-A-9304194	04-03-93	US-A- EP-A- JP-T-	5434050 0599979 6510761	18-07-95 08-06-94 01-12-94
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